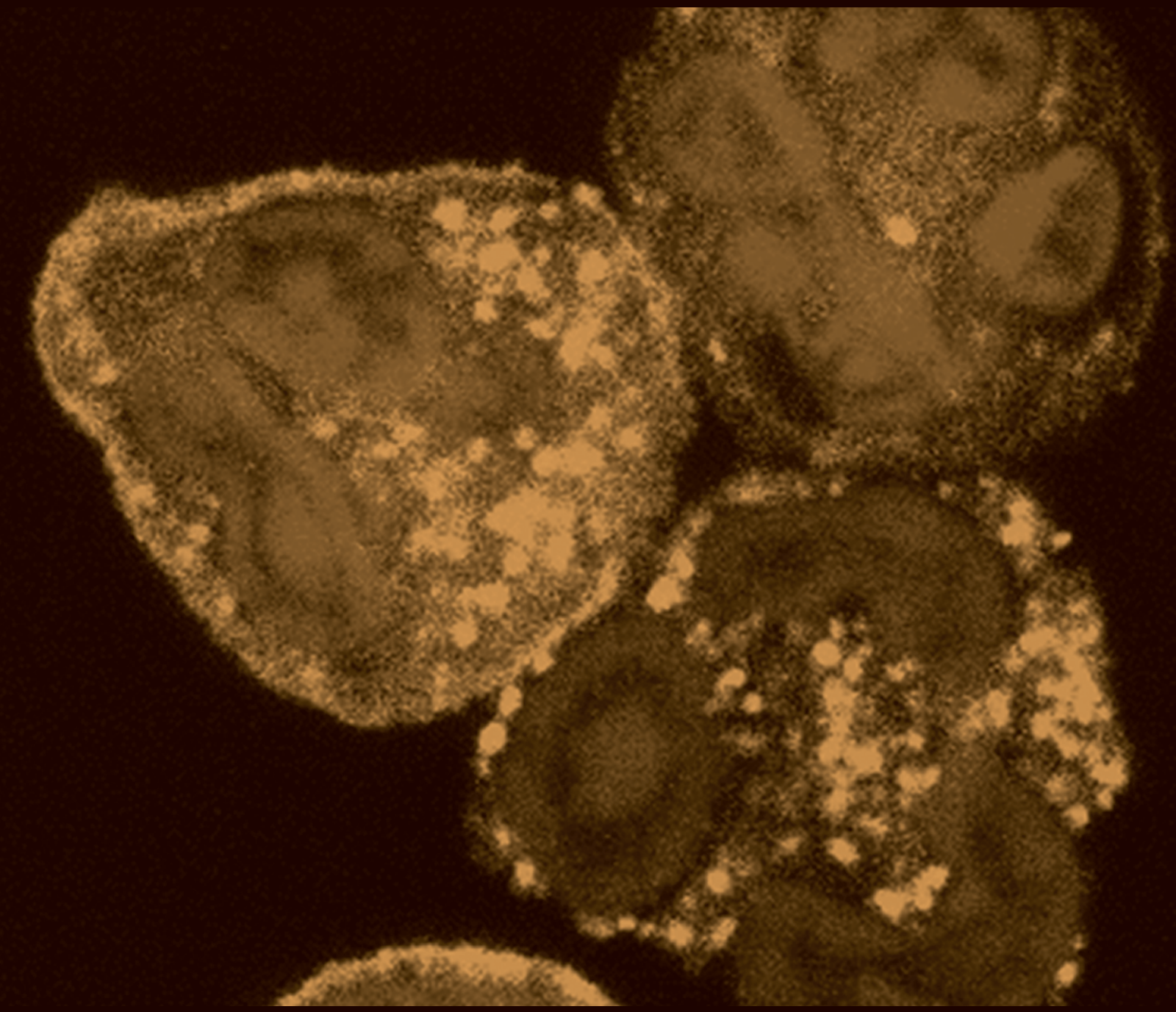
Mediators of Inflammation in Obesity and Its Comorbidities

Guest Editors: Oreste Gualillo, Giamila Fantuzzi, Gema Frühbeck, Giuseppe Matarese, and Paul Trayhurn



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Editorial

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In the last decades we have seen a rapid expansion of the proportion of obese individuals worldwide. Actually obesity has gradually but also rather considerably revealed as a whole medical problem with menacing implications for the health of our society. Indeed, obesity is already of epidemic proportions in the U.S. and in many other parts of the world and it is a worrying problem also in developing countries. According to the last data of World Health Organization, more than 1 billion people are overweight worldwide, where more than 300 million fulfil obesity criteria [1]. Obesity is accompanied by a plethora of other relevant diseases including cardiovascular, cerebrovascular, and liver diseases, type 2 diabetes, and dyslipidemias that are affecting also youngest age groups of population. Obesity has been recently recognized also as a relevant contributing risk factor for certain types of cancer [2].

Emerging literature suggests that inflammation, as evaluated by high inflammatory cytokines levels and other inflammatory markers, may represent basically a cause and consequence of obesity and its comorbidities [3]. We have to bear in mind that obesity is a consequence of millions of years of evolution during which the ability to store efficiently fat in long periods of fluctuation of food availability, alternated by famines, granted capacity to survive, reproduce, and maintain survival of offspring. In the last 50 years, characterized by an excess of food availability, this adaptive skill shifted to maladaptive increasing the propensity to obesity and its comorbidities above mentioned. Several disorders that represent the major sources of morbidity and mortality in the present world such as type 2 diabetes, cardiovascular disease, and cancer are known to be associated with obesity and have recently been reconsidered as inflammatory diseases. For instance, the inflammation associated with the accumulation of intra-abdominal fat is associated with progressive resistance to the effects of insulin, ultimately leading to type 2 diabetes [4]. Anyway, the causes for the activation of the inflammatory response in obesity and its comorbidities are undoubtedly complex and we are in the beginning of the road to cover. Indeed, increasing evidence (most of them reported in this special issue) suggests a possible causal link between adiposity, particularly visceral obesity and inflammation. Obesity leads to increases in inflammatory cytokines and adipocytokines and changes in related molecules such as leptin and adiponectin, which may contribute to the development of multiple disturbs in predisposed individuals. However, in most cases the relationship appears to be bi-directional, in that prior comorbidities seem to increase and perpetuate the proinflammatory status associated to adiposity. Clearly, more research is needed to examine the complex interplay between inflammation and adiposity, an effect that is also in part to consequences of low physical activity. However, the interactions between these systems offer unique opportunities for targeted treatment and prevention strategies.

For instance, there is a pressing need to investigate strategies to disrupt inflammatory signalling in persons with obesity (morbid or not) who show indication of a proinflammatory state (e.g., those with elevated CRP). Research on approaches that alter diet, increasing the intake of particular micronutrients, or, of course, reducing weight, is also needed [5].

This special issue has focused on adiposity and inflammation as one of the main contributing factors for obesity-associated morbidities.

However, several salient facts must be noted. Obesity and comorbidities are complex and multifaceted diseases

with too many contributing factors. So, here we intended to suggest that inflammation and adiposity contribute strongly in all or even most affected people. The prevention of obesity would significantly reduce the burden of comorbid diseases. Indeed, by decreasing prevalence of obesity, other obesity-related conditions can be reduced to a greater extent and this will help in cutting down the health care budget to a great extent (in times of economic crisis as such we are living everywhere, this should be mandatory considered).

One year ago approximately, I answered to a call of our Editor in Chief, Professor Freek Zijlstra, suggesting a special issue on mediators of inflammation in obesity and its comorbidities, and he has indeed selected a worthy topic.

In this issue of Mediators of Inflammation, we are pleased to present to the reader a series of special features written by great authorities in the field. In this special issue, the reader will find several articles (more than 25) written by experts on epidemiology, mechanisms and molecules, regulation of body weight, concomitants of obesity, and therapeutic approaches to obesity.

I would like to thank all contributors and reviewers and I am personally grateful for your support to this special issue as there can be no growth or improvement without your participation.

Finally, a special thank is also due to the Associate Editors of this special issue Giamila Fantuzzi, Gema Fruhbeck, Giuseppe Matarese, and Paul Trayhurn for their hard work, commitment, and support.

Oreste Gualillo

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Review Article

Peritoneal Adipocytes and Their Role in Inflammation during Peritoneal Dialysis

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Adipose tissue is a major site of chronic inflammation associated with peritoneal dialysis (PD) frequently complicating peritonitis. Adiposity-associated inflammation plays a significant contributory role in the development of chronic inflammation in patients undergoing maintenance PD. However, the molecular and cellular mechanisms of this link remain uncertain. Adipose tissue synthesizes different adipokines and cytokines that orchestrate and regulate inflammation, insulin action, and glucose metabolism locally and systemically. In return, inflammation retards adipocyte differentiation and further exacerbates adipose dysfunction and inflammation. An understanding of the inflammatory roles played by adipose tissue during PD and the healing mechanism of injured mesothelium will help to devise new therapeutic approach to slow the progression of peritoneal damage during peritoneal dialysis. This article reviews the roles of peritoneal adipose tissue in chronic peritoneal inflammation under PD and in serosal repair during PD.

1. Introduction

Continuous ambulatory peritoneal dialysis (CAPD) has emerged as a major treatment modality in renal replacement program worldwide. It has distinct advantages over hemodialysis with a lower cost and simplicity of the technique. The ability to maintain the functional integrity of the peritoneal membrane allowing effective removal of fluid and metabolic waste is essential for the success of the treatment. Unfortunately, the peritoneal membrane frequently exhibits structurally changes following long-term dialysis due to the exposure of unphysiologic peritoneal dialysis fluid (PDF) with low pH and high glucose [1]. PDF also contains toxic substances including glucose degradation products (GDP) generated during the sterilization process and advanced glycation end products (AGE) produced from Amadori reaction between sugar and protein during long-term peritoneal dialysis (PD) [2]. These compounds cause irreversible damage to the peritoneal tissue leading to ultrafiltration failure and decline in dialysis efficacy [3, 4]. Previous studies have reported the detrimental effects of PDF on peritoneal cells including human peritoneal mesothelial cells (HPMC) [5–7] and endothelial cells [8, 9]. While adipose tissue is ubiquitously present in peritoneal tissue, information for the characteristics and pathophysiology of adipocytes following long-term exposure to PDF in maintenance CAPD remains scarce. Only until recently, adipocytes are considered as passive tissue for the storage of energy in the form of fat. However, there are now compelling evidences suggesting that adipocytes exert important metabolic and proinflammatory effects on peripheral tissue [10–12]. Furthermore, peritoneal adipocytes affect HPMC through the release of adipokines and, hence, alter the peritoneal physiology during PD [13, 14].

2. Peritoneal Adipocytes

The parietal and visceral peritoneal surfaces are covered by a monolayer of mesothelium composed of mesothelial cells. Beneath the mesothelial cells are the basement membrane and submesothelial layer that contains collagen, fibroblasts, adipose tissue, blood vessels, and lymphatics [15]. Adipose tissue is abundant in omental or mesenteric peritoneum but less so in parietal, intestinal, and diaphragmatic peritoneum.

Contrary to the prevailing view that adipose tissue functions only as an energy storage depot, compelling evidence reveals that adipocytes can mediate various physiological processes through secretion of an array of mediators and adipokines that include leptin, adiponectin, resistin, tumor necrosis factor-α (TNF-α), interleukin (IL)-6, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and other growth factors [16]. Moreover, adipocytes express receptors for leptin, insulin growth factor-1 (IGF-1), TNF-α, IL-6, TGF-β and may form a network of local autocrine, paracrine, and endocrine signals [17]. All of these adipokines exert important endocrine functions in chronic kidney diseases and may also contribute to systemic inflammation in these patients. This is of special significance in patients undergoing CAPD as the initiation of treatment is often associated with an increase in fat mass that could be associated with a polymorphism in uncoupling protein 2 which affects the energy metabolism in addition to glucose absorption from the PDF [18]. In contrast to findings in the general population, a number of studies have suggested that a higher body mass index (BMI) is associated with a better outcome in patients with kidney diseases [19]. Critical analysis reveals that the protective effect from a high BMI only applies to patients with a normal or high muscle mass [20]. A recent study indicates that an increased fat mass in PD, like in other patient groups, may indeed have adverse metabolic consequences with increased systemic inflammation and worst survival [21]. Interestingly, there is a difference in the release of growth factors between visceral and subcutaneous adipose tissue [22]. The omental adipose tissue, most affected by PD, releases IL-6 two to three folds higher than the subcutaneous fat tissue [23]. The visceral (truncal) fat mass correlates significantly with circulating IL-6 levels but not for nontruncal fat mass [24].

Ultrastructural study reveals that a portion of omental adipocytes protrude from the mesothelial surface, thus may come into direct contact with dialysate [15]. In addition, dialysate may also reach the parietal adipose tissue when the mesothelial monolayer is damaged. It is therefore logical to postulate that with repeated exposure to PDF and the continuous change in peritoneal physiology during CAPD, peritoneal adipocytes will inevitably be "activated". Although much work has focused on peritoneal mesothelial cells, scant attention has been paid to the role of peritoneal adipocytes during CAPD.

3. Stem Cells from Adipose Tissue

The stromal vascular fraction (SVF) is a heterogeneous cell population derived from the adipose tissue including omentum [25–27]. SVF is reported to be composed of endothelial cells identified as CD34+/CD31+ cells, infiltrating/resident macrophages defined as CD14+/CD31+ cells, and a population characterized as CD34+/CD31− cells. The CD34+/CD31− subset is a unique cell fraction capable of differentiating into adipocytes and is restricted to cells that do not express the mesenchymal stem cell marker CD105 [28]. It has been suggested that the adipocyte progenitor cells, that is, the preadipocytes, are included in the CD34+/CD31− cell fraction. This unique population is distinct from the multipotent adipose tissue-derived mesenchymal stem cells, which can be differentiated in vitro into other cell types including adipocytes [27], chondrocytes [29], osteoblasts [30, 31], and cardiomyocytes [32, 33]. The cellular number of SVF varies among individuals and so far there is no data studying whether PD alters the number of SVF in different adipose depots. Apart from the SVF, milky spots of the omentum also harbor stem cells [34], which proliferate to form the resident macrophage during peritoneal inflammation [35]. It remains unknown whether stem cells from milky spots have the same identity as stem cells in SVF with adipogenic potential. Milky spots are very small omental tissues in contact with peritoneal membrane, consisting of macrophages, lymphocytes, and plasma cells supported by blood and lymphatic vessels. Milky spots play a role in peritoneal infection and abdominal tumors [36, 37]. PD also activates the milky spots resulting in an increase in number and size during inflammatory process and PD [37, 38]. Milky spots transform into a lymph node-like structure where lymphocytes constitute the main cellular component after an episode of peritonitis [34].

4. Crosstalk between Peritoneal Cells and Adipocytes with a Focus on Leptin

Adipose tissues express and secrete a variety of cytokines and adipokines, which act locally as autocrine/paracrine mediators or systemically as endocrine factors (Table 1). Patients on PD have increased fat mass due to glucose absorption from the PDF. Increase in adiposity has been associated with sub-clinical inflammation with elevated adipokines synthesis. Among these adipokines, leptin is of particular interest as this peptide hormone is most abundant adipokine produced by adipocytes and is cleared principally by the kidney. The serum leptin concentration is increased in patients with chronic renal failure or undergoing dialysis [39, 40] and the serum leptin increases by 189% within a month after the initiation of PD treatment [41]. Leptin is also elevated during acute infection, in response to proinflammatory cytokines including IL-1 and TNF-α [39]. In the kidney, leptin stimulates cell proliferation and synthesis of collagen IV and TGF-β in glomerular endothelial cells. In glomerular mesangial cells, leptin increases the glucose transport, upregulates the expression of the TGF-β type II receptor and the synthesis of collagen I through phosphatidylinositol-3-kinase related pathway [39]. Available data suggests that leptin triggers a paracrine interaction between glomerular endothelial and mesangial cells through the increased synthesis of TGF-β in glomerular endothelial cells and upregulated TGF-β receptor expression in mesangial cells. It remains unclear whether such paracrine interaction operates between peritoneal adipocytes and HPMC. To the best of our knowledge, there is only one previous study on the effect of PDF on adipocytes that demonstrates increased leptin

Table 1: Major adipokines and cytokines released from adipose tissue.

Adipokine/cytokine	Cellular source in adipose tissue	Inflammatory effect	Relevance to PD	References
Leptin	Adipocytes	Pro-inflammatory	Serum and dialysate leptin increased after PD Leptin augmented myofibroblastic conversion of HPMC	[14, 59–61]
Adiponectin	Adipocytes	Antiinflammatory	Glucose-based PDF increased plasma leptin/adiponectin Level in PD patients may indicate of cardiovascular disease risk	[62–64]
Resistin	Macrophages Adipocytes	Pro-inflammatory	Level correlates with fat mass and triglycerides in PD patients	[64–66]
Visfatin	Macrophages Adipocytes	Pro-inflammatory	Serum visfatin levels were higher in the PD patients	[67]
RBP-4	Adipocytes	Pro-inflammatory	RBP-4 is significantly increased in end-stage renal disease	[68, 69]
NGAL	Mesothelial cells Adipocytes	Pro-inflammatory	Prolonged release of NGAL in dialysate following peritonitis NGAL was proposed as a novel early marker for acute renal failure	[52, 53]
TNF-α	Adipocytes Macrophages Mesothelial cells Endothelial cells	Pro-inflammatory	TNF-α production by macrophage was reduced by low pH and lactate in PDF Adipose-derived TNF-α inhibited leptin production	[60, 70, 71]
IL-6	Macrophages Adipocytes Mesothelial cells Endothelial cells	Pro-inflammatory	Plasma and dialysate IL-6 were associated with high peritoneal solute transport rate Mesothelial cells released IL-6 upon exposure to the spent dialysate or IL-1β	[72–74]
Apelin	Adipocytes	Pro-inflammatory	TNF up-regulated apelin expression in adipose tissue	[71, 75]
MCP-1	Macrophages Adipocytes Preadipocytes	Pro-inflammatory	MCP-1 was up-regulated by TNF-α and regulated the differentiation of adipocytes	[76–78]

synthesis in a murine adipocyte cell line (3T3-L1) by glucose-containing PDF [42]. It is likely that pro-inflammatory mediators released by HPMC upon exposure to PDF could induce functional alteration of adjacent adipocytes. The likely candidates are IL-1 and TNF-α, TGF-β, VEGF, and IL-6. Indeed, a recent in vitro study has shown that IL-6 modulates leptin production and lipid metabolism in human adipose tissue [43]. Using cultured HPMC and SVF, we have shown that high glucose content in dialysate fluid is one of the major culprits that causes structural and functional abnormalities in peritoneal cells during CAPD [13, 44, 45]. Glucose significantly increases the protein synthesis of leptin by adipocytes in a dose-dependent manner and up-regulates the expression of leptin receptor, Ob-Rb, in HPMC [13]. The increased leptin production by adipocytes and enhanced Ob-Rb expression in HPMC following exposure to glucose suggest the existence of a cross-talk mechanism between adipocytes and mesothelial cells that may be relevant in peritoneal membrane dysfunction developed during peritoneal dialysis. HPMC cultured with conventional PDF induce higher expression of VEGF than that experiments with low-GDP-content PDF. In parallel, GDPs increase the gene and/or protein expression of VEGF in HPMC [46]. GDPs also decrease the expression of proteins associated with the tight junction, zonula occludens protein 1 (ZO-1), in HPMC [44]. Exogenous VEGF down-regulates the expression of ZO-1 while neutralizing anti-VEGF antibody reverses the effect of GDPs on ZO-1 expression in HPMC. These findings suggest that the action of GDPs on ZO-1 expression is mediated through VEGF.

A longitudinal study conducted in patients treated for PD-related peritonitis revealed elevation of serum leptin levels during acute peritonitis. The rise was contributed to anorexia in the earlier stage. In contrast, the serum adiponectin levels fell showing an inverse correlation between these two adipokines during acute peritonitis. Furthermore, the protracted course of inflammation even after bacterial cure of peritonitis was likely to cause the loss of lean body mass and to increase mortality [47].

5. Persistent Release of Pro-Inflammatory Mediators in Patients under Maintenance PD or after an Episode of Peritonitis

Patients on maintenance PD have increased intraperitoneal levels of hyaluronan and cytokines including IL-1β, IL-6, and TGF-β [48, 49]. Chronic inflammation remains an important cause of morbidity in patients with end-stage renal failure. The main causes for inflammation in CAPD are PD-related peritonitis and exit site infection [50]. Patients on PD with peritonitis may experience prolonged inflammation even when clinical evaluation suggests resolution of PD-related peritonitis [51]. The highly sensitive C-reactive protein (hs-CRP) remains significantly higher than baseline even by day 42 after an episode of peritonitis [47]. There was persistent release of Neutrophil Gelatinase-Associated Lipocalin (NGAL) in the peritoneal dialysate effluent (PDE) collected following an acute episode of CAPD-related peritonitis. NGAL synthesis is specifically induced in HPMC by IL-1β during peritonitis [52]. Interestingly, NGAL is also produced by adipocytes [53]. NGAL markedly affects the secretion of leptin and adiponectin by adipocytes, and acts as a negative regulator of inflammatory activity and inflammation-mediated adipocyte dysfunction. Incubation of HPMC with recombinant NGAL reverses the up-regulation of Snail and vimentin induced by TGF-β. Our data suggest that NGAL exerts a protective effect by modulating the epithelial-to-mesenchymal transition activated by peritonitis [52].

6. Role of Stem Cells from Adipose Tissue in Serosal Repair during CAPD

It has been shown that daily instillation of PDF for 5 weeks in rats leads to an increased number of omental mast cells and milky spots as well as damage to the mesothelial cell layer covering the peritoneum membrane [54]. Most interestingly, electron microscopy reveals that the severely damaged mesothelial cells are able to regenerate a good monolayer upon three months' rest of the peritoneum. The exact mechanism regulating this reversibility is not completely understood. Adipose tissues-derived SVF contains pluripotent mesenchymal stem cells that can regenerate damaged tissue [55]. An abundance of progenitor cells is also found in omentum [56]. Introduction of a foreign body into the peritoneal cavity further enhances the healing capability of the omentum by causing it to expand, surround the foreign body, and transform itself from mostly fatty tissue [56]. This transformed tissue (the activated omentum) contains abundant progenitor cells positive for CXCR-4 or Wilm's tumor-1 (WT-1), and is also rich in growth and angiogenic factors [56]. Activated omentum also facilitates liver regeneration following traumatic injury [57]. SVF cultured from omentum expresses pluripotent markers, produces high amounts of VEGF, and engrafts to injured sites [58]. These observations support a regenerative potential of mesothelium although the underlying mechanism remains undefined. The relative contribution of mesothelial cells, SVF or adipocytes in the adipose tissue and the relevant mechanism involved in the healing process of mesothelium after CAPD have not been well characterized.

During peritoneal dialysis, the undesirable microenvironment, chronic inflammation, and previous peritonitis all impose stress, causing damage to the peritoneal membrane. Remesothelialization or healing is possible if the peritoneum is allowed to rest [54]. Regeneration or healing of the mesothelium does not occur solely by centripetal migration of cells from the wound edge. It has been proposed that pluripotent cells beneath the mesothelium migrate towards the surface and differentiate into mature mesothelial cells [79–81]. Others suggest that the new mesothelium originates from a free-floating mesothelial cell or progenitors in the serosal fluid [82]. Different origins of cells in the regenerating mesothelium have been proposed and these include subserosal mesenchymal precursors, bone marrow-derived precursors, free-floating macrophages, and free-floating mesothelial cells. The exact identity of this cell population responsible for mesothelial repair remains uncertain.

Normal stem cells, mobilized from the bone marrow or resident in damaged tissue, play a pivotal role in tissue regeneration or healing after injury [83]. The α-chemokine stromal-derived factor-1 (SDF-1) and its unique G-protein-coupled chemokine receptor (CXCR4) constitute the SDF-1/CXCR4 axis that regulates the trafficking of stem cells during the repair of damaged tissues. SDF-1 is involved in the regulation of CXCR4+ progenitor cell trafficking [84–86]. Proper functioning of the SDF-1/CXCR4 axis plays a pivotal role in the healing and regenerative processes of damaged tissue [87], and this may be relevant to the repair of peritoneal membrane after CAPD. Accumulation of these progenitor cells in peritoneal tissues is affected by a cascade of inflammatory mediators produced by peritoneal cells (including macrophages, mesothelial cells, endothelial cells, and adipocytes) following long-term exposure to PDF during peritoneal dialysis. Unpublished data from our laboratory and from the literature [88, 89] demonstrate that SDF-1, CXCR4, as well as the endogenous aminopeptidase dipeptidyl peptidase IV (DPPIV or CD26 that controls the degradative pathway of the SDF-1) are expressed by HPMC (Figures 1(a) to 1(d), unpublished data). Notably, peritoneal permeability in CAPD patients with frequent peritonitis deteriorates with parallel increased expression of TGF-β in dialysate [90]. The SDF-1 expression is up-regulated in damaged tissue following TGF-β treatment leading to an increased migratory potential of CXCR4 bearing cells (including HPMC and progenitor cells from the bone marrow or adipose tissue) to the SDF-1-positive niche [89]. Other cytokines including HGF and VEGF may also participate in the up-regulation of SDF-1 synthesis in injured tissue. Up-regulation of the SDF-1 expression implicates the reepithelialization of denuded basement membrane at the site of peritoneal injury. This hypothesis is supported by the observation of a time- and dose-dependent reduction of DPPIV and E-cadherin expression in HPMC following TGF-β-induced morphological change. Following the inhibition of DPPIV, degradation of CXCR4 is retarded and hence significantly enhances the migratory potential of CXCR4

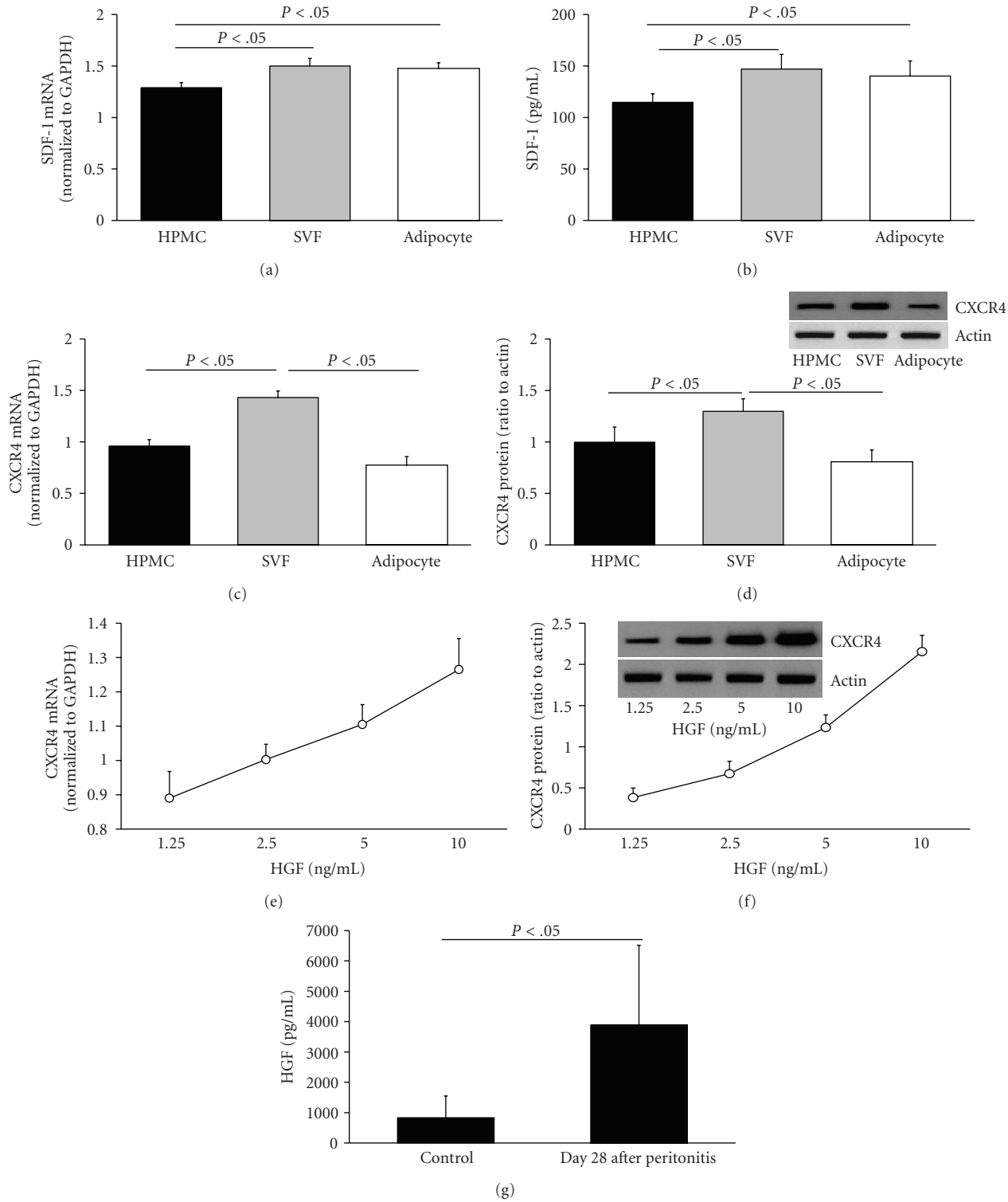

Figure 1: Constitutive expression of mRNA (expressed as amplicon ratio after normalized to GAPDH, measured by quantitative PCR), protein for G-protein-coupled chemokine receptor (CXCR4; expressed as ratio of densitometry data after normalized to GAPDH, measured by immunoblotting), and stromal derived factor-1 (SDF-1; measured by ELISA) in cultured human peritoneal mesothelial cells (HPMC), stromal vascular fraction (SVF), and adipocytes from human omental tissue (a to d). The CXCR4 expression in HPMC was up-regulated in a dose-dependent manner with hepatocyte growth factor (HGF) after 4 hours culture (e and f). Overnight PD effluent fluid ($n = 15$) was collected from CAPD patients on day 28 after the onset of peritonitis. Control PD effluent fluid ($n = 15$) was obtained in CAPD patients without previous history of peritonitis. The concentration of the HGF in PD effluent fluid was measured by ELISA. Persistent release of HGF in PD effluent was observed at day 28 after peritonitis in CAPD patients (g). These data are from our unpublished studies.

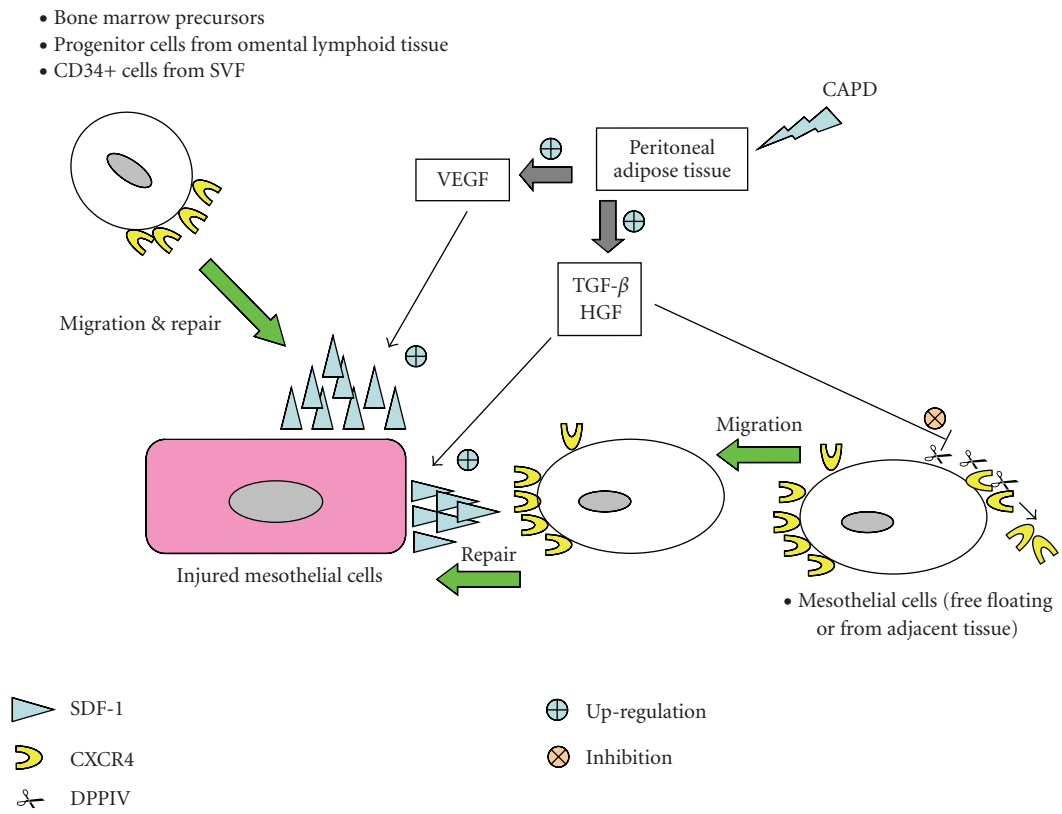

Figure 2: Schematic model illustrates the roles of adipokines or cytokines from adipose tissue on the repair of mesothelium under the context of CAPD. DPPIV indicates aminopeptidase dipeptidyl peptidase IV; CXCR4, G-protein-coupled chemokine receptor; SDF-1, stromal derived factor-1; SVF, stromal vascular fraction; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

positive HPMC towards the SDF-1 gradient in the injured tissue. Apart from TGF-β, HGF also affects the SDF-1/CXCR4 axis. HGF increases the CXCR4 expression and SDF-1 production in glioma and facilitates their invasion [91, 92]. HGF released during peritonitis also alters mesothelial cell phenotype and function [93]. We observe a dose-dependent up-regulation of CXCR4 expression in HPMC by HGF (Figures 1(e) and 1(f), unpublished data). The dialysate level of HGF remains persistently elevated even 28 days after an episode of peritonitis (Figure 1(g)). The pleiotropic HGF may initially affect the mesothelial healing by promoting mesothelial cell growth, but can also contribute to peritoneal fibrosis by stimulating cell detachment with mesothelial denudation and collagen synthesis [93, 94]. The pathophysiological impact of prolonged release of these pro-inflammatory mediators on the SDF-1/CXCR4 axis and the mesothelial healing remains to be examined. Figure 2 is a schematic outlining the potential role of peritoneal adipokines and their interplay with the SDF-1/CXCR4 axis in regulating the regeneration process of the mesothelium in CAPD.

7. Conclusion

Long-term peritoneal dialysis is often associated with structural alterations of the peritoneal membrane that are closely related to chronic local as well as systemic inflammatory responses. It is evident that peritoneal mesothelial cells, fibroblasts, and macrophages exert their effects on peritoneal membrane during PD. Increasing evidences reveal that peritoneal adipose tissue also plays an important role in the structural and functional alterations during PD. In particular, adipocytes release secretory adipokines and cytokines that play modulating roles in the inflammatory cascade and healing response of the mesothelium in PD. In the present review, we summarized the relevance of adipose tissue associated adipokines and cytokines in PD, with focuses on recent data related to the leptin synthesis by peritoneal adipocytes and the associated cellular crosstalk with mesothelial cells. The possible involvement of the SDF-1/CXCR4 axis and adipose tissue-derived mediators in the regeneration process of the injured mesothelium after PD was also discussed. In order to better preserve the integrity of the peritoneal membrane, which facilitates long-term CAPD, novel studies designed to elucidate the detailed interaction between different peritoneal cellular components with the adipocytes in the context of PD should be undertaken. Further studies on the identity of peritoneal progenitor cells and the precise role of the SDF-1/CXCR4 axis in maintaining the peritoneal membrane function for peritoneal dialysis are warranted.

Abbreviations

AGE:	Advanced glycation end products
DPPIV or CD26:	Aminopeptidase dipeptidyl peptidase IV
BMI:	Body mass index
CAPD:	Continuous ambulatory peritoneal dialysis
GDP:	Glucose degradation products
CXCR4:	G-protein-coupled chemokine receptor
HGF:	Hepatocyte growth factor
hs-CRP:	Highly sensitive C-reactive protein
HPMC:	Human peritoneal mesothelial cells
IGF-1:	Insulin growth factor-1
IL:	Interleukin
NGAL:	Neutrophil Gelatinase-Associated Lipocalin
PDE:	Peritoneal dialysate effluent
PD:	Peritoneal dialysis
PDF	Peritoneal dialysis fluid
SDF-1:	Stromal derived factor-1
SVF:	Stromal vascular fraction
TGF-β:	Transforming growth factor-β
TNF-α:	Tumor necrosis factor-α
VEGF:	Vascular endothelial growth factor
WT-1:	Wilm's tumor-1
ZO-1:	Zonula occludens protein 1.

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Review Article

Release of Inflammatory Mediators by Human Adipose Tissue Is Enhanced in Obesity and Primarily by the Nonfat Cells: A Review

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This paper considers the role of putative adipokines that might be involved in the enhanced inflammatory response of human adipose tissue seen in obesity. Inflammatory adipokines [IL-6, IL-10, ACE, TGFβ1, TNFα, IL-1β, PAI-1, and IL-8] plus one anti-inflammatory [IL-10] adipokine were identified whose circulating levels as well as in vitro release by fat are enhanced in obesity and are primarily released by the nonfat cells of human adipose tissue. In contrast, the circulating levels of leptin and FABP-4 are also enhanced in obesity and they are primarily released by fat cells of human adipose tissue. The relative expression of adipokines and other proteins in human omental as compared to subcutaneous adipose tissue as well as their expression in the nonfat as compared to the fat cells of human omental adipose tissue is also reviewed. The conclusion is that the release of many inflammatory adipokines by adipose tissue is enhanced in obese humans.

1. Introduction

There is increasing evidence that obesity in humans is associated with low-level inflammation [1–6] that is often accompanied by hypertension and type 2 diabetes. Currently it is thought that the increase in visceral omental rather than abdominal subcutaneous adipose tissue best correlates with measures of insulin resistance [7] and cardiovascular disease [8–10]. However, the amount of visceral fat has an allometric relationship with total body fat content [11] which means that the increases in visceral fat mass seen in obesity reflect the initial ratio of visceral fat to total fat mass as well as the changes in total fat mass change. Thus during weight loss or gain there are concurrent changes in the amount of both subcutaneous and visceral fat.

The distribution of fat between premenopausal men and women is different with women having generalized lipid deposition as contrasted to men who tend to accumulate fat in the abdominal region resulting in a socalled "beer belly". There are also sex differences in the ratio of visceral to abdominal subcutaneous fat mass between men and women [4]. The visceral fat mass of the women was approximately 50% of the abdominal subcutaneous fat mass while for the men it was 98% [4].

The measurement of abdominal subcutaneous and visceral fat mass can be done using either a computed tomography (CT) or MRI scan. Measurement of total body fat requires either a DXA scan or a bioelectrical impedance scale. In contrast, waist circumference is simply measured and provides as good if not better measure of the health risks of obesity than the more complex procedures [12, 13]. However, the use of BMI has the advantage of comparing men and women on the same scale since it is an index of weight corrected for height.

This review will primarily discuss studies on the effects of obesity on circulating adipokines, the relative release of adipokines by the fat cells versus the nonfat cells of human adipose tissue, the effects of obesity on adipokine release by explants of human visceral omental adipose tissue, and the differences in gene expression between visceral and subcutaneous fat. The term adipokine, as used in this review, means any protein released by adipose tissue without regard to whether it is released by the fat or the other cells (nonfat cells) found in human adipose tissue.

2. Effects of Obesity on Circulating Levels of Adipokines

At least 24 adipokines have been reported whose circulating levels are elevated in obese humans (Table 1). Some of these putative adipokines such as CRP, haptoglobin, and amyloid A are actually acute phase proteins primarily released by the liver in response to the mild inflammatory response seen in human obesity. Most of the remaining 21 are inflammatory proteins such as IL-8, PAI-1, MCP-1, IL-6, IL-1Ra, TNFα, sTNF RII, and IL-18 but the source of the elevated circulating levels in obesity is unclear. Their elevations could result from release by tissues other than fat. In contrast, leptin levels are elevated in obesity and the current paradigm is that it is released by fat cells in adipose tissue. However, in mice it has been shown that activated T cells and other lymphocytes can also release leptin under inflammatory conditions [14, 15].

The circulating levels of zinc-α2-glycoprotein (ZAG) have been reported to be unaltered in obesity [17], but the level of ZAG gene expression in human adipose tissue is reduced in obesity [69, 70]. This illustrates the problem that changes in circulating levels of adipokines do not necessarily reflect changes in their release by or correlate with their mRNA levels in adipose tissue. Most of the adipokines are also cytokines and are released primarily by cells other than fat cells in human adipose tissue (Figure 1). Furthermore, circulating levels of all adipokines are also regulated by their release from other tissues as well as their degradation. For others such as interleukin 1β (IL-1β), no reports have been published indicating that IL-1β is elevated in the circulation of obese humans. However, IL-1β is an important regulator of the inflammatory response in human adipose tissue. It may well be a paracrine regulator that acts locally and never reaches the blood in mild inflammatory conditions such as obesity. The same may apply to PGE_2, which is the primary product of the cyclooxygenase-2 (COX-2) enzyme.

Some of the adipokines may actually have anti-inflammatory effects and circulate at higher levels in obesity as part of a homeostatic mechanism to counteract the effects of the inflammatory mediators. Probably interleukin 10 (IL-10) is such a molecule [71] and there is some evidence that interleukin 6 (IL-6) has dual effects since it has been claimed that it enhances insulin action in muscle [72]. Interestingly there is also evidence that administration of a meal enhanced release of IL-6 by human adipose tissue perfused in situ [73]. It is as yet unclear whether IL-6 is enhancing or inhibiting insulin action but the traditional view is that IL-6 inhibits insulin action [74].

While 24 putative adipokines are listed in Table 1 whose circulating levels are elevated in obesity there are only two out of 37, adiponectin and glutathione peroxidase 3 (GPX-3), whose circulating levels have been reported to be lower in human obesity. The current paradigm is that circulating levels of adiponectin are reduced in obesity [25, 33, 34]. However, the finding that circulating GPX-3 is also lower [35], if confirmed, suggests that GPX-3 may also be important. GPX-3 is unique among the five known isoforms of this enzyme since it is the only one that is secreted by cells [75]. It is a selenocysteine-containing protein with antioxidant properties. The circulating levels of GPX-3 and selenium have also been reported to be lower in patients with coronary artery disease than in age-matched controls [76].

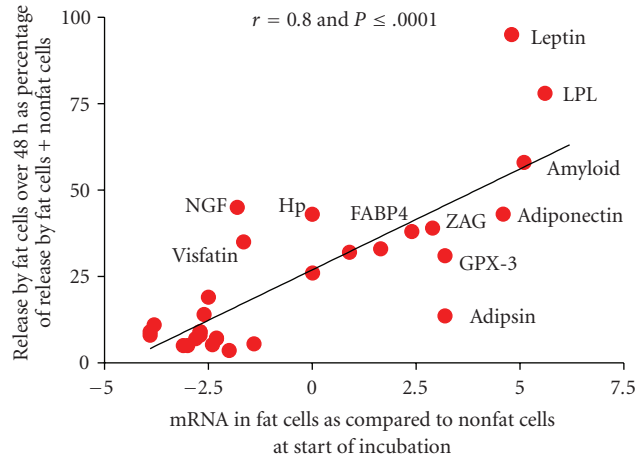


Figure 1: The correlation between releases of 30 adipokines over 48 hours incubation by fat cells isolated from human adipose tissue as compared to gene expression of these adipokines at the start of the incubation. The release data are from Table 1 and expressed as release by fat cells as % of that by fat cells plus nonfat cells over 48 hours. The data for mRNA are derived from those shown in Table 2 except that they are plotted as the ΔCp for the difference between mRNA in fat cells and nonfat cells instead of the ratios, which are derived from the ΔCp values. Data are not included for resistin, CRP and IL-18 since release by fat cells was below the sensitivity of the assays and mRNA was not measured for MIF, HGF, VEGF, and VCAM-1.

3. The Relative Release of Adipokines by the Nonfat versus the Fat Cells of Human Adipose Tissue

It has often been assumed that release of an adipokine by adipose tissue is due to the fat cells. This originated with the finding by Rodbell [77] that lipoprotein lipase [LPL] is localized in the fat cells of rat adipose tissue. It was in order to solve the problem of the localization of LPL that Rodbell [78] developed the collagenase procedure for separation of insulin-responsive fat cells from the nonfat cells in rat adipose tissue. However, Cleland et al. [79] found that most of the aromatase activity in human adipose tissue, that is responsible for estrogen formation from androstenedione, was localized in the nonfat cells and most of the IL-6 release by human adipose tissue was by the nonfat cells [80]. Fain et al. [62] subsequently reported on the relative release of 11 adipokines by the nonfat as compared to the fat cells of human omental and abdominal subcutaneous adipose tissue during an in vitro incubation. Leptin was found to be released exclusively by the fat cells, while TNFα, hepatocyte growth factor (HGF), IL-10, IL-1β, PGE_2, IL-6, vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8) were primarily released by the nonfat cells.

Table 1: Comparison of release of 37 adipokines by fat cells as compared to the other cells in human adipose tissue ranked by fat cell release along with the effect of obesity on their circulating levels in humans.

Adipokine	Release by nonfatcells in pmoles/g	Release by fatcells in pmoles/g	Effect of obesity on circulating levels
FABP-4	**590**	**360**	**Elevated [16–18]**
PGE_2	1810*	118*	No data
IL-8	**1120***	**87***	**Elevated [21, 22]**
PAI-1	**78***	**18***	**Elevated [19, 23, 24]**
MCP-1	**74***	**9.2***	**Elevated [4, 20, 22, 25]**
IL-6	**66***	**5.1***	**Elevated [4, 25–31]**
Adipsin	**26**	**4.1**	**Elevated [28, 32]**
Adiponectin	*6*	*4.1*	*Lower[25, 33, 34]*
GPX-3	*14*	*3.6*	*Lower [35]*
Leptin	**0.1**	**1.8**	**Elevated [19, 25, 36]**
Amyloid A	**1.3**	**1.6**	**Elevated [19, 32, 37]**
Migration inhibitor factor	**2.8**	**1.0**	**Elevated [38]**
Visfatin/PBEF/Nampt	1.0	0.60	No change [39–41]
CD14	1.6	0.56	No change [42]
ZAG	0.7	0.44	No change [17]
Lipocalin-2	5.4	0.27	No change [42, 43]
Cathepsin S	**4.4**	**0.26**	**Elevated [44]**
RANTES	2.1	0.21	No change [42] but see [25]
IL-1Ra	**4.1**	**0.14**	**Elevated [36]**
Osteoprotegerin	0.1	0.12	No change [42, 45]
HGF	**2.8**	**0.11**	**Elevated [46]**
LPL	0.02	0.080	No change [47]
Haptoglobin	**0.08**	**0.060**	**Elevated [48]**
ICAM-1	**0.27**	**0.056**	**Elevated [4, 29, 49]**
ACE	**0.23**	**0.017**	**Elevated [50]**
IL-10	**0.53**	**0.020**	**Elevated [28]**
VEGF	**0.30**	**0.020**	**Elevated [51]**
VCAM-1	**0.46**	**0.016**	**Elevated [29]**
IL-1β	0.23	0.013	No data
TNFα	**0.22**	**0.012**	**Elevated [5, 27, 29–31, 52]**
TGF-β1	**0.17**	**0.009**	**Elevated [53]**
sTNF RII	**0.44**	**0.007**	**Elevated [5, 18, 19, 30, 54]**
NGF	**0.006**	**0.005**	**Elevated [55]**
VEGFR/sFLT1	0.018	0.002	No change [34]
Resistin	1.8	<0.04	No change [56, 57]
CRP	**0.01**	**<0.002**	**Elevated [17, 19, 20, 28, 30, 31, 49, 58]**
IL-18	**0.01**	**<0.002**	**Elevated [18, 59, 60]**

Those in "bold" are adipokines whose circulating levels have been reported to be elevated in obesity, "italic" those where the circulating levels are lower in obesity, and "normal text" where there is either no effect of obesity or published data. The references are to the reports on circulating levels. The asterisks indicate that the release of these adipokines was almost certainly upregulated over 48 hours. The rate of release for IL-8 over 48 hours extrapolated from release over the first 40 minutes of incubation were 2.2% of those based on the 48 hours release values [61]. The release values for nonfat and fat cells over 48 hours are the averages of subcutaneous and omental adipose tissue from 8 to 12 humans with a BMI of 32 and an equal number with a BMI of 45. These data are from Fain et al., [62] except for haptoglobin [63], resistin [64], MCP-1 [65], TGFβ1 [66], MIF, Cathepsin S, NGF, IL-1Ra, IL-18 [67], VCAM-1, ACE, adipsin, sTNFR2 [68], CD14, LPL, OPG, Amyloid A, ZAG, GPX-3, FABP-4, ICAM-1, RANTES, visfatin, lipocalin-1 [42] while CRP and VEGFR/sFLT1 are from unpublished experiments.

In vitro, the relative release of adipokines by fat cells as compared to nonfat cells derived from human adipose tissue over a 48 hours incubation indicates that the highest release by fat cells was of fatty acid binding protein 4 (FABP-4) followed by IL-8 (Table 1). The high value for IL-8 release over 48 hours is primarily due to upregulation, since the rate of release over 48 hours derived from release during the first 40 minutes was only 2% of the 48 hours release value for both fat cell and nonfat cells [61]. Adipokine release was up-regulated to the same extent in both types of cells of either omental or subcutaneous fat [61].

The question arises as to how well in vitro release of adipokines over the first 48 hours of primary culture by human fat cells and nonfat cells reflects the in vivo situation. That cannot be determined because it takes a two-hour digestion to separate fat cell from nonfat cells and during that time there is upregulation of the mRNAs for inflammatory cytokines such as IL-8 and IL-6 [81]. However, what can be measured is the level of gene expression in the nonfat cells versus the fat cells at the start of the incubation which can be compared to release over 48 hours. These data are shown in Figure 1 for 30 of the 37 adipokines shown in Table 1. There was an excellent correlation (Pearson correlation coefficient of 0.8) between release of adipokines over 48 hours by fat cells as % of that by nonfat cells and the initial ratio of the mRNA for the adipokine in fat cells versus nonfat cells. The data also demonstrate that leptin release is exclusively by the fat cells of omental adipose tissue, which also contained 28-fold more leptin mRNA than the nonfat cells (Figure 1). Release of LPL was also primarily by the human fat cells and in agreement with the 79-fold greater amount of its mRNA found in fat cells as compared to nonfat cells.

Adiponectin has generally been considered to be an adipokine released exclusively by fat cells but while the ratio for mRNA expression in fat cells as compared to nonfat cells was 42-X the release of adiponectin accounted for only 40% of total release. Fain et al. [82] suggested that immature fat cells or other cells in the nonfat cell fractions of human adipose tissue also release adiponectin. Alternatively, the release could be due to adiponectin taken up by nonfat cells in vivo and then released during the 48 hours incubation. The release of amyloid A by human fat cells as % of that by nonfat cells was actually higher than that of adiponectin and its mRNA content in fat cell was 34-fold greater than that in nonfat cells. However, amyloid, like adiponectin, release appears to be about the same by nonfat as by fat cells. While leptin, LPL, amyloid A, and adiponectin are adipokines predominantly expressed in fat cells at ratios 30 to 80-fold greater than in nonfat cells (Figure 1), the question of whether there is appreciable amyloid and adiponectin synthesis by the nonfat cells of adipose tissue remains to be established.

There are four other possible candidates for the designation of adipokines preferentially released by fat cells, since the ratios of their mRNAs in fat cells to nonfat cells ranged from 5 for FABP-4, 8 for ZAG, and 9 for adipsin/complement D as well as GPX3. However, release by fat cells accounted for less than half of their total release.

4. Relative Expression of 100 Genes in Fat Cells versus the Nonfat Cells of Human Omental Adipose Tissue

Table 2 shows the relative gene expression in fat cells versus nonfat cells of 100 proteins, as determined by qRTPCR [83]. These proteins were chosen because they are important in inflammation or obesity, regulatory proteins or proteins enriched in fat cells.

Of the proteins whose gene expression is shown in Table 2 almost one-third (30) were significantly enriched in fat cells (shown in Bold), 29 were distributed equally (shown in italic) and 41 were significantly enriched in nonfat cells of human omental adipose tissue (shown in normal text). Thirty of these proteins are the adipokines whose release by adipose tissue was examined in the studies shown in Table 1 and Figure 1.

Of special interest was the finding that 11β HSD1, UCP-2, cyclic AMP phosphodiesterase 3B, AQP7, angiotensinogen, GPX-3, the insulin receptor, and NQO1 are preferentially localized in fat cells [83]. Interestingly ZAG, TLR4, cytochrome C oxidase, Akt2, adrenomedullin, and UCP-1 were also expressed at levels 4 to 8-fold greater in fat cells than in nonfat cells [Table 2]. The higher expression of ZAG in human fat cells than in nonfat cells confirms the report by Bao et al. [84].

An elevated expression in fat cells was seen for both cytochrome C oxidase, which is a marker for mitochondria, and Akt2, which is the isoform of Akt involved in insulin-stimulated glucose uptake into fat cells [85]. The enhanced expression of the mitochondrial protein UCP-1 in visceral omental fat cells was unexpected since it is thought of as a marker for brown fat cells. However, Sacks et al. [86] found far higher expression of UCP-1 in visceral epicardial fat as compared to subcutaneous fat. The increased expression of cytochrome C oxidase in fat cells as compared to nonfat cells of omental fat suggests that fat cells are relatively enriched in mitochondria. Deveaud et al. [87] have shown that cytochrome C oxidase is enriched in visceral epididymal fat of rats as compared to subcutaneous inguinal fat.

The circulating levels of adrenomedullin are elevated in human obesity [88, 89]. Furthermore, adrenomedullin is secreted by fat cells [90, 91] but it is unclear whether more adrenomedullin is secreted by fat cells than by the nonfat cells of human adipose tissue [88–91].

The proteins whose gene expression was predominantly in the nonfat cells included all the classical inflammatory proteins such as MCP-1, TGFβ1, IL-6, IL-8, COX-2, PAI-1, IL-1β, IL-8, and TNFα (Table 2). Other putative adipokines, such as vaspin, endothelin-1, omentin/intelectin, lipocalin-2, RANTES, and visfatin were also enriched in the nonfat cells. Vaspin is an adipose tissue-derived serpin whose gene expression in human visceral fat positively correlated with obesity [92]. Circulating levels of omentin/intelectin are lower in obesity [93] but the meaning of this is unclear.

The ratio of gene expression in fat cells to nonfat cells ranged from 0.06 to 128 (Table 2). However, if in vitro differentiated human omental adipocytes were compared to omental preadipocytes the ratios ranged from 0.001 to over a million for adiponectin [82, 83]. Clearly there is more expression of fat cell specific proteins in freshly isolated nonfat cells than in preadipocytes obtained by culturing the nonfat cells of human omental fat. This difference may be accounted for, in part, by the presence of small fat cells without enough fat to float, since isolated fat cells are operationally defined as cells containing enough lipid to float in isotonic incubation buffer. Another possibility is incomplete digestion of adipose tissue leaving some fat cells

Table 2: Comparison of 100 mRNAs in fat cells as compared to the nonfat cells derived from human omental adipose tissue.

	Ratio of mRNA in fat cells to nonfat cells	Cp value in nonfat cells
mRNAs significantly enriched in fat cells		
Perilipin	**128**	**29.3**
Hormone sensitive lipase [HSL]	**104**	**32.4**
Lipoprotein lipase [LPL]	**79**	**26.5**
Adiponectin	**42**	**28.1**
Retinol binding protein 4 [RBP-4]	**42**	**29.4**
Adipose tissue triglyceride lipase [ATGL]	**37**	**35.4**
Amyloid protein A1	**34**	**27.3**
Leptin	**28**	**29.2**
FAT/CD36	**26**	**25.8**
11β-hydroxysteroid dehydrogenase 1 tv1 [11β HSD-1]	**18**	**30.8**
PPARγ	**15**	**30.4**
Uncoupling protein 2 [UCP-2]	**14**	**28.9**
Fat specific protein 27/CIDEC	**13**	**26.0**
CIDEA	**12**	**27.0**
Glutathione peroxidase 3 [GPX-3]	**9**	**27.0**
Adipsin/complement D	**9**	**27.9**
Zinc α_2-glycoprotein [ZAG]	**8**	**28.8**
Cyclic AMP phosphodiesterase 3B	**7**	**27.6**
Angiotensinogen	**5**	**34.1**
Toll-like receptor 4 [TLR-4]	**5**	**34.8**
Fatty acid binding protein 4 [FABP-4]	**5**	**20.2**
Cytochrome c oxidase	**4**	**28.1**
Glycerol channel aquaporin 7 [AQP-7]	**4**	**27.4**
Adrenomedullin	**4**	**26.4**
Akt2/protein kinase B2	**4**	**27.2**
Uncoupling protein 1 [UCP-1]	**4**	**32.6**
NADPH:quinone oxidoreductase l [NQO-1]	**3**	**27.2**
Insulin receptor tv1 [INSR]	**3**	**27.5**
GAPDH	**3**	**26.7**
CGI-58/ABHD5	**3**	**26.2**
mRNAs present in both nonfat cells and fat cells		
Giα2 guanine nucleotide binding protein	*2.1*	*32.0*
Osteoprotegerin [OPG]	*1.9*	*31.0*
Thrombospondin 1	*1.8*	*24.5*
Sodium hydrogen exchanger 1	*1.6*	*28.9*
AMPK α2 catalytic subunit	*1.5*	*32.9*
Akt/1protein kinase B1	*1.5*	*27.4*
Lipin-1	*1.5*	*26.5*
Lipin-2	*1.5*	*27.4*
Cyclophilin A	*1.4*	*29.0*
Caveolin-1	*1.4*	*26.6*
MAP3K8/COT1	*1.3*	*26.5*
Receptor interacting protein 140 [RIP 140]	*1.3*	*24.6*
Haptoglobin	*1.0*	*31.2*
SIRT1/sirtuin1	*1.0*	*27.1*
CD14 tv1	*1.0*	*26.0*
IL-1 Ra	*1.0*	*33.3*
Leucine-rich protein PPR [LRP130]	*0.87*	*27.0*

Table 2: Continued.

	Ratio of mRNA in fat cells to nonfat cells	Cp value in nonfat cells
$NFKB_1$ [p50]	*0.81*	*33.0*
β2 adrenergic receptor	*0.76*	*25.8*
Hypoxia inducible factor 1α [HIF-1α]	*0.71*	*27.4*
CD 68	*0.71*	*24.0*
RAB6	*0.66*	*25.0*
Peroxisome proliferator activator receptor-γ coactivator 1α [PGC-1]	*0.57*	*30.0*
Apelin	*0.57*	*33.1*
MAP4K4 tv 2	*0.57*	*29.2*
Heme oxygenase-1 [HMOX-1]	*0.57*	*24.4*
Renin receptor	*0.54*	*25.8*
β1 adrenergic receptor	*0.50*	*27.8*
Resistin	*0.33*	*33.6*
mRNAs significantly enriched in nonfat cells		
Endothelial nitric oxide synthase [eNOS]	0.44	30.2
PI-3 kinase catalytic subunit	0.38	27.2
Cathepsin S	0.38	31.4
NFKB p65	0.35	29.2
Mitochondrial superoxide dismutase-2 tv1 [SOD2]	0.35	21.0
Tumor necrosis factor-α receptor 2 [TNFR-2]	0.29	31.2
Nerve growth factor beta polypeptide [NGF]	0.29	31.5
Interleukin 10 [IL-10]	0.25	30.0
Visfatin/PBEF/Nampt	0.25	21.9
PR domain containing 16 [PRDM16] tv1	0.25	31.5
Tumor necrosis factor α [TNFα]	0.25	30.5
Glycogen synthase kinase 3β	0.23	32.0
Interleukin 8 [IL-8]	0.20	23.6
Osteocalcin	0.20	30.2
α1 glycoprotein	0.20	33.8
Complement C-3	0.19	25.9
Interleukin 1β [IL-1β]	0.19	25.7
Prostaglandin D_2 synthase [PGDS]	0.18	23.9
Tribbles 3 [TRB3]	0.18	30.6
Plasminogen activator inhibitor 1 [PAI-1]	0.18	27.3
Bone morphogenetic protein 7 [BMP-7]	0.16	31.8
Intercellular adhesion molecule 1 [ICAM-1]	0.16	25.2
Cyclooxygenase 2 [COX-2]	0.15	27.4
RANTES	0.15	30.0
Angiotensin 1 converting enzyme [ACE]	0.15	31.0
Interleukin 6 [IL-6]	0.14	25.0
Endocannabinoid receptor 1	0.14	25.8
Collagen type VI/PBEF1α3 tv1 [COL6-A3]	0.14	29.9
NADPH oxidase $p67^{phox}$	0.13	30.5
TGFβ-1	0.12	27.3
NADPH oxidase $p47^{phox}$	0.12	32.4
Lipocalin 2	0.12	33.5
Butyrylcholinesterase	0.11	33.2
Angiotensin II receptor-1 tv1 [AT_1R]	0.10	25.7
Omentin/intelectin	0.09	25.0
Monocyte chemoattractant protein 1 [MCP-1]	0.07	23.2
VEGF receptor [VEGFR/FLT1]	0.07	27.8

Table 2: Continued.

	Ratio of mRNA in fat cells to nonfat cells	Cp value in nonfat cells
25-Hydoxyvitamin D3 1α hydroxylase [25D3-1α]	0.06	26.8
Endothelin-1	0.06	27.8
Angiotensin II receptor-2 [AT_2R]	0.06	33.1
Vaspin	0.06	28.1

Those in "bold" are the mRNAs significantly enriched in fat cells, "italic" those mRNAs present in both nonfat cells and fat cells to the same extent, and "normal text" those mRNAs significantly enriched in nonfat cells. The data are based on quantitative PCR analysis of mRNA expression [14, 82, 83]. The ratios were derived from the $\log_2\Delta\Delta$Cp of the ΔCp (crossing point) for each mRNA, except cyclophilin, of the nonfat cells (pooled undigested tissue + SV fractions) obtained by collagenase digestion of human omental adipose tissue subtracted from the ΔCp values of fat cells isolated from the same tissue. The ratio for cyclophilin is based on $\log_2$ of the ΔCp values for cyclophilin. A ratio above 1 means that the amount of mRNA is greater in the fat cells than in the nonfat cells. The values are shown as the means ± SEM of 4 to 21 paired experiments comparing nonfat cells to fat cells derived from the same individual. Tv1 or 2 stands for transcript variant 1 or 2. The data are from Fain et al. [82, 83] or from unpublished data.

entrapped in the undigested tissue matrix. However, if this is the case these cells secrete very little leptin since its release by the nonfat cell fraction is less than 5% of that by isolated fat cells (Figure 1) and we could find no detectable fat in the nonfat cells [67].

One problem in comparing gene distribution between fat and nonfat cells is the possibility of preferential lysis of extremely large fat cells during the collagenase digestion of fat from extremely obese humans. The isolation of human fat cells is an art requiring particular batches of collagenase for optimal yield of responsive cells, gentle incubation conditions and an optimal ratio of collagenase to tissue [62, 67]. Fain et al. [67] calculated that there was a 23% greater loss of fat cells during digestion than of nonfat cells during the digestion of fat from extremely obese humans. The fat cells lost during digestion may well be the largest fat cells that release more inflammatory adipokines and leptin than the smaller cells. A further problem is the up-regulation of inflammatory response genes during the 2 hours required for collagenase digestion but this affects both fat cells and nonfat cells to the same extent [61] and thus has minimal effects on the ratios of mRNA expression in fat to nonfat cells.

5. Comparison of mRNA Expression in Isolated Omental Fat Cells versus In Vitro Differentiated Adipocytes

Many studies on the relative gene expression of proteins in fat cells have utilized adipocytes differentiated in vitro such as murine 3T3L1 cells, but far fewer studies have appeared using human cell lines. The term fat cells is operationally defined as those cells that float and are isolated by collagenase digestion of human omental adipose tissue from women undergoing bariatric surgery. Adipocytes are those fat cells derived from the adipose tissue of the same group of women that underwent differentiation in vitro in the presence of insulin, dexamethasone, a methyl xanthine, and a thiazolidinedione.

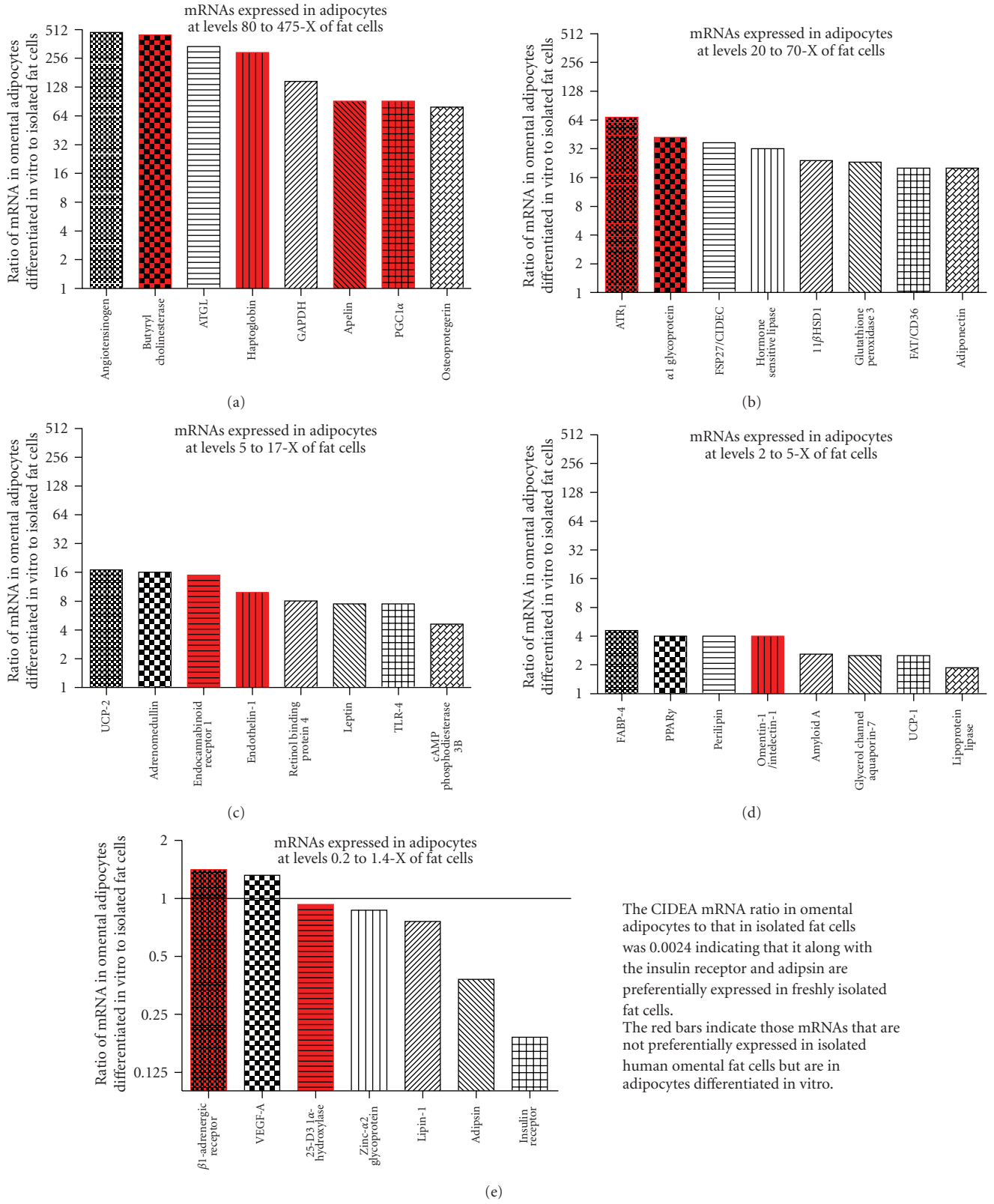
In the data shown in Figure 2 the mRNA content of freshly isolated omental fat cells versus in vitro differentiated adipocytes was compared using total RNA as the recovery standard as suggested by Bustin [94] since the expression of cyclophilin A used as the recovery standard differed significantly between fat cells and in vitro differentiated adipocytes. The data indicate that many proteins are expressed at far higher levels in adipocytes than in freshly isolated fat cells. Some proteins that are expressed at higher levels in adipocytes than in fat cells are not enriched in freshly isolated fat cells as compared to nonfat cells (Figure 2). These are shown in red and are: butyryl cholinesterase, haptoglobin, apelin, PGC1α (peroxisome proliferator activator receptor-γ coactivator 1α), ATR_1 (angiotensin II receptor 1), αl glycoprotein, endocannabinoid receptor 1, endothelin-1, and omentin/intelectin.

Five mRNAs were found at comparable levels in adipocytes as compared to fat cells. These were the β1 adrenergic receptor, 25-hydroxyvitamin D3 1α hydroxylase, VEGF-a, ZAG, and lipin-1. Three genes were expressed at lower levels in adipocytes than in fat cells: adipsin, insulin receptor, and CIDEA. The data suggest that the one or more of the added factors required for differentiation of preadipocyes to adipocytes induce the expression of many proteins that are not induced in vivo and decrease the expression of others such as CIDEA and the insulin receptor. Clearly the use of human adipocytes differentiated in vivo from preadipocytes does not result in a pattern of gene expression comparable to that seen in intact fat from obese women.

6. Effect of Obesity on In Vitro Adipokine Release by Explants of Human Adipose Tissue

Studies using freshly isolated explants preserve the cross talk between the various types of cells in fat. However, since the primary effect of obesity is to increase adipose tissue mass, it is difficult to know how to express data obtained by primary culture of human fat explants. How do you compare total release by adipose tissue from humans with 20 kg of fat as compared to those with 40 kg? In the studies shown in Figure 3 release in vitro over a 48 hours incubation of omental and subcutaneous fat from each woman per kg of fat was multiplied by the total fat content. The women were then divided by tertiles based on body fat content.

There was enhanced release of endothelin-1, lipocalin-2, visfatin, GPX-3, and FABP-4 by the most obese women as compared to that by women in the bottom tertile (Figure 3).



The CIDEA mRNA ratio in omental adipocytes to that in isolated fat cells was 0.0024 indicating that it along with the insulin receptor and adipsin are preferentially expressed in freshly isolated fat cells.
The red bars indicate those mRNAs that are not preferentially expressed in isolated human omental fat cells but are in adipocytes differentiated in vitro.

Figure 2: Comparison of mRNA expression in isolated omental fat cells versus in vitro differentiated adipocytes. The data are shown as the ratio of mRNA in human omental adipocytes, differentiated in vitro from the nonfat cells isolated from omental adipose tissue, to that in freshly isolated fat cells obtained by collagenase digestion of omental adipose tissue from female bariatric surgery patients. The ratios were derived from the Cp values and plotted on a $\log_2$ scale. Comparable amounts of total RNA were used for the mRNA analyses. The Cp values from which the ratios were determined for fat cells were calculated from the data shown in Table 2 and for in vitro differentiated adipocytes from Fain et al. [82, 83] or unpublished data. The red bars are for mRNAs whose expression in isolated fat cells was either the same or lower than in isolated nonfat cells.

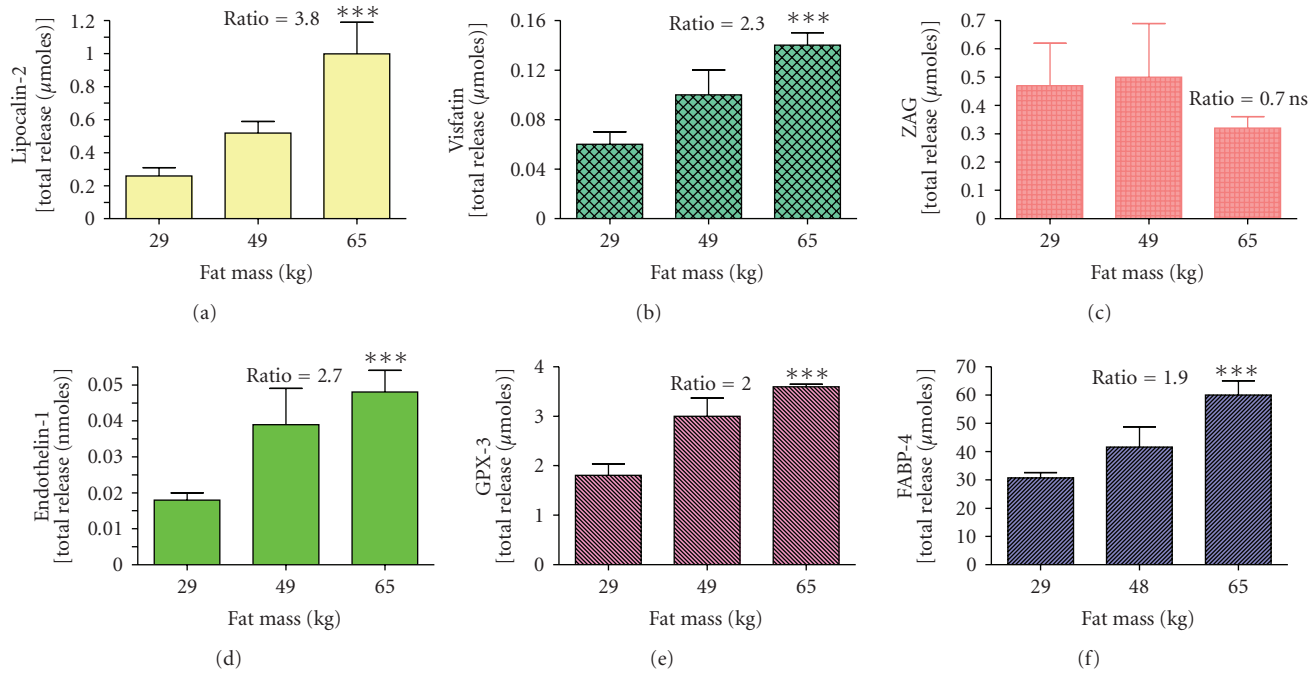


Figure 3: The effect of obesity on total release of 6 adipokines by explants of adipose tissue from obese women. The data are from the report by Fain et al. [42] for release of 6 adipokines by adipose tissue from 22 women divided into tertiles. The lowest tertile was composed of 7 women with total fat masses of 18 to 40 kg with a mean of 29 kg. The middle tertile was composed of fat from 8 women with total fat masses ranging from 41 to 52 kg with a mean of 49 kg. The highest tertile was fat from 7 women with fat masses ranging from 56 to 75 kg (mean of 65 kg). The ratio of total release by the highest tertile as compared to the lowest tertile is shown and all ratios were significant with a $P < .001$ except for zinc $\alpha 2$ glycoprotein [ZAG] release that was not statistically significant ($P > .05$).

For ZAG we found no effect of obesity since total release was not significantly higher in women in the highest tertile but they had 124% more fat than women in the lowest tertile. Therefore there was actually decreased release per g of adipose tissue. This is in agreement with reports that gene expression of ZAG in fat is reduced in human obesity [69, 70]. There was enhanced total release of intercellular cell adhesion molecule 1 (ICAM-1), CD14, and LPL but not of osteoprotegerin, RANTES or amyloid A [42].

Another way to examine the effect of obesity is to correlate total release with the total fat mass of each woman. That resulted in a correlation coefficient for lactate release of 0.81 and for IL-8 release of 0.85 based on total release plotted against the fat mass of each woman (Figure 4). A positive correlation indicates that the more fat you have the greater the total amount of lactate or IL-8, if release per g of fat remains the same. In contrast, total amyloid and VEGF release did not correlate with total fat mass indicating that their release per g of tissue was less but the total release by fat remained constant.

Data for 24 other adipokines are summarized in Table 3, along with those for lactate, amyloid A, and VEGF and IL-8 release shown in Figure 3. Adipokines that showed no correlation, that is, those whose total release actually decreased in obesity, were MCP-1, interleukin 1 receptor antagonist 1 (IL1-Ra), adipsin, osteoprotegerin, RANTES, ZAG, cathepsin S, vascular cell adhesion cell molecule 1 (VCAM-1) and NGFβ in addition to VEGF and amyloid A. A number of inflammatory adipokines had a significant correlation between total release and total fat mass besides IL-8 and these included, IL-10, transforming growth factor $\beta 1$ (TGF$\beta 1$), visfatin, IL-1β, IL-6, CD14, endothelin-1, ICAM-1, TNFα, lipocalin-2, PAI-1, and angiotensin 1 converting enzyme (ACE) that are primarily released by the nonfat cells. There was also a significant correlation between total release and fat mass for FABP-4, GPX-3, and LPL.

A problem complicating release studies by human fat is that incubation in vitro induced an inflammatory response as judged by enhanced mRNA accumulation over the 48 hours incubation for IL-8, IL-10, TGF$\beta 1$, visfatin, IL-1β, IL-6, ICAM-1, TNFα, lipocalin-2, PAI-1 and ACE (Table 3). Interestingly, an increase in mRNA expression over 48 hours was seen for MCP-1, osteoprotegerin, and NGFβ whose total release was not enhanced by obesity. Furthermore there was no significant change in the mRNA expression over 48 hours of CD14, endothelin-1 or ACE while there was a marked decrease in FABP-4, GPX-3, and LPL mRNA but enhanced release in obesity. These data suggest that the in vitro inflammatory response does not mimic completely the effect of obesity.

In conclusion, adipose tissue from extremely obese women, when incubated in vitro, releases more of a host of adipokines such as IL-8, IL-10, TGF$\beta 1$, visfatin, IL-1β, IL-6, ICAM-1, TNFα, lipocalin-2, PAI-1, and ACE than does tissue from women with a lesser amount of fat. While TNFα appears to be important it is one adipokine whose

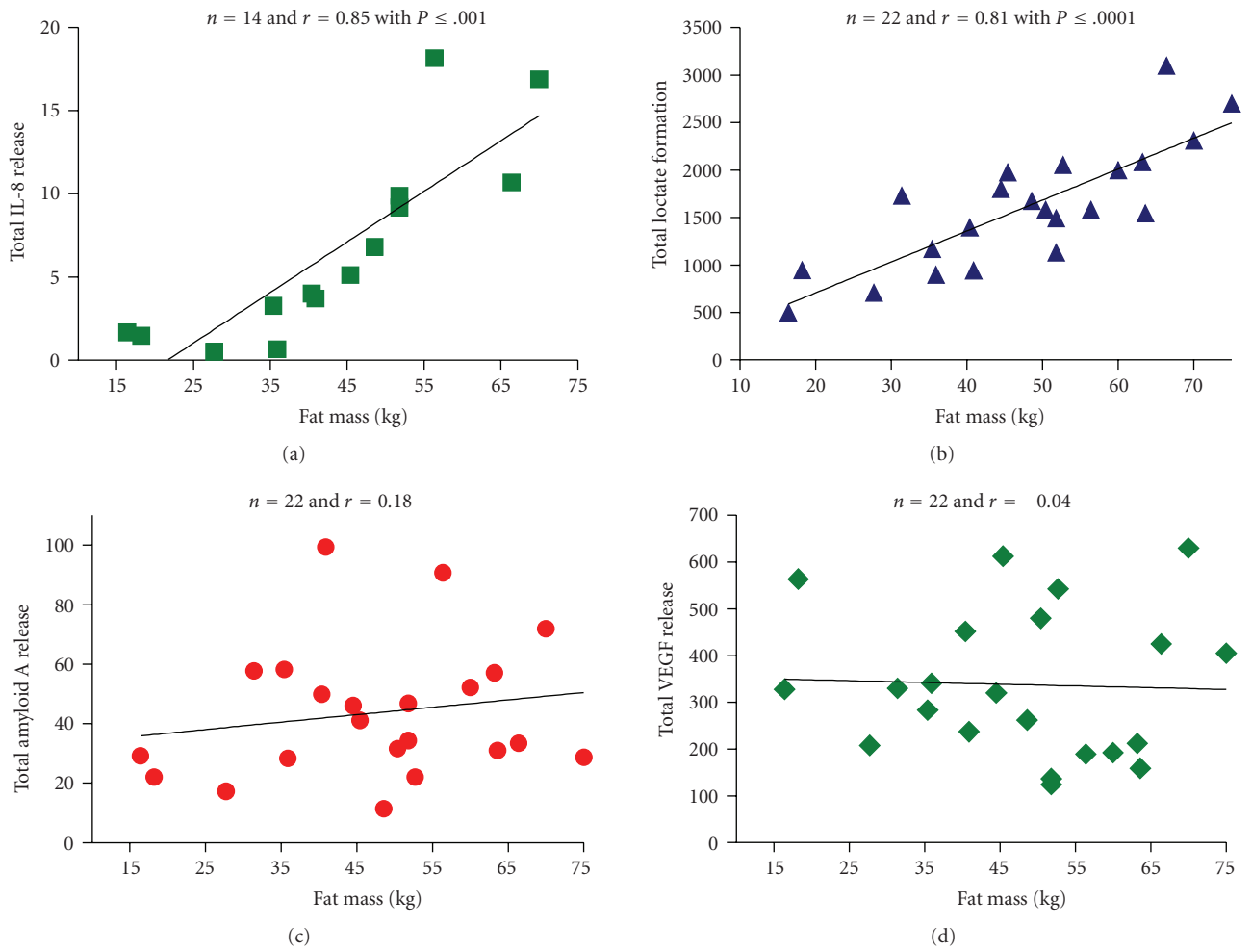


Figure 4: Correlation between total release of IL-8, VEGF, Amyloid A, and lactate by adipose tissue and total fat mass. The total release was calculated by averaging release over 48 hours per kg by explants of visceral omental and subcutaneous adipose tissue from 14 [IL-8] or 22 different women (lactate, amyloid A and VEGF) and multiplying by the total fat mass. Tissue samples were from the same women described by Fain et al. [42]. The Pearson correlation coefficients (r) are shown on the figure and the P value if statistically significant with a $P < .05$.

mRNA and release goes up transiently during in vitro incubation of adipose tissue, but unlike other members of the inflammatory cascade its release and gene expression return to near basal values by 48 hours [61, 96].

7. Which Cells in the Nonfat Cell Fraction Derived from Human Adipose Tissue Are Responsible for Release of Inflammatory Adipokines?

Hellman et al. [97] reported in 1963 that obesity in the obese-hyperglycemic mouse resulted in greater accumulation of mast cells in white adipose tissue. They also pointed out that the relative nitrogen content per gram of the epididymal fat pad of the obese-hyperglycemic mouse was unchanged despite the marked reduction in the number of fat cells per g of tissue. Almost 40 years later Xu et al. [98] extended this to show that the expression of genes enriched in murine macrophages such as MCP-1, TNFα, CD68, and F4/80 was elevated in obese mice. They also demonstrated that all of these genes were preferentially expressed in the nonfat cells of murine white fat [98]. Weisberg et al. [99] independently published similar findings and emphasized that the size of fat cells positively correlated with the percentage of macrophages in murine adipose tissue.

Subsequently it was demonstrated that HAM56+ macrophage accumulation in visceral omental and subcutaneous fat depots of humans also positively correlated with the diameters of the fat cells in each depot. However, at any fat cell size there were more macrophages in omental than subcutaneous fat despite the fact that the average diameter of subcutaneous fat cells was 40% greater than that of omental fat cells [100]. The use of HAM56 as the macrophage marker is important since in humans CD68 [101, 102], CD14 [102], or F4-80 [102] are much less specific macrophage markers than in mice. Similar results are shown in Table 2 in that the gene expression of both CD14 and CD68 was not significantly different between the fat cells and nonfat cells isolated from human omental adipose tissue.

Table 3: Correlation between total release by explants of human fat and total fat mass as well as the change in mRNA over 48 hours incubation.

Adipokine	r value for correlation of release with fat mass	Change in mRNA over 48 hour (ratio)
Those with significant positive correlations of total release with fat mass		
IL-8	**0.85**	**510**
Lactate	0.81	
FABP-4	0.73	0.06
IL-10	**0.70**	**13**
TGFβ1	**0.69**	**8**
Visfatin	**0.67**	**30**
IL-1β	**0.65**	**120**
IL-6	**0.65**	**675**
CD14	0.64	1.7
Endothelin-1	0.63	1.4
sICAM-1	**0.61**	**6.1**
TNFα	0.59	
GPX-3	0.56	0.33
Lipocalin-2	**0.54**	**34**
PAI-1	**0.53**	**295**
ACE	0.52	0.55
LPL	0.51	0.14
Those with no significant correlations of total release with fat mass		
Amyloid A	0.18	0.50
MCP-1	**0.12**	**37**
IL-1 Ra	**0.10**	**9**
Adipsin	0.01	0.23
Osteoprotegerin	**0.01**	**4.9**
VEGF	−0.04	
RANTES	−0.05	1.2
Cathepsin S	−0.07	
ZAG	−0.09	0.25
VCAM-1	−0.30	
NGFβ	**−0.30**	**13**

The values shown in bold are for adipokines whose gene expression was upregulated over the 48 hours incubation. The changes in mRNA as measured by qRTPCR over 48 hours were based on comparison of unincubated adipose tissue explants with those after 48 hours and shown as the ratios derived from the ΔCp values [95]. The Pearson correlation coefficient (r) was derived by plotting the total release over 48 hours by the average of values for omental and subcutaneous adipose tissue versus the calculated total fat mass in 20–22 obese women as described in Fain et al. [66–68]. The correlation coefficients have been published for TGFβ1 [66], cathepsin S, nerve growth factor β (NGFβ), interleukin-1 receptor antagonist (IL-1Ra) and interleukin 18 (IL-18) [67] as well as those for adipsin, vascular cell adhesion molecule 1 (VCAM-1), and angiotensin1 converting enzyme (ACE) [68]. The correlation coefficients for the following are derived from Fain et al. [42]: endothelin-1, zinc-α2-glycoprotein (ZAG), lipocalin-2, CD14, RANTES, lipoprotein lipase (LPL), osteoprotegerin, fatty acid binding protein 4 (FABP-4), visfatin, glutathione peroxidase-3 [GPX-3], intracellular cell adhesion molecule 1 [ICAM-1], and amyloid A while those for IL-8, IL-6, PAI-1, TNFα, IL-10, VEGF, and IL-1β were derived from Fain et al. [62]. The changes in mRNA over 48 hours are from Fain et al. [95].

The current paradigm is that obesity results in accumulation of macrophages in adipose tissue and these are primarily responsible for the release of inflammatory mediators [98–100]. A relevant question is whether macrophages are the only mononuclear phagocytes found in adipose tissue and whether they account for all of the adipokine release by nonfat cells. The potential contribution of the other nonfat cells in human adipose tissue such as the endothelial cells of the blood vessels, the smooth muscle cells and fibroblasts as well as other mononuclear phagocytes has not been carefully examined.

Why do macrophages localize in the white adipose tissue of obese animals? Whether enhanced lysis/death of large fat cells is the primary trigger that accounts for inflammation is unknown as well as what signal results in greater macrophage accumulation in adipose tissue. One of the functions of macrophages is to aid in the clearing of dead cells. Cinti et al. [103] suggested that macrophages are localized selectively to sites of necrotic-like cell death where they appear as crown-like structures when viewed in tissue sections. They also suggested that fat cell hypertrophy per se promotes cell death resulting in macrophage accumulation and aggregation around dead cells. The current paradigm is that the larger the fat cell the more likely it is to undergo cell death. However, a consistent finding is that human visceral omental fat cells are smaller than subcutaneous fat cells

from the same individual but the macrophage accumulation is greatest in omental fat so something besides fat cell size is important [104]. Furthermore, thiazolidinediones appear to selectively enhance the breakdown of large fat cells in visceral omental fat resulting in smaller more insulin-sensitive fat cells [105]. The net effect of thiazolidinediones is to preferentially enhance deposition of fat in subcutaneous adipose tissue while decreasing that in visceral fat [105].

8. The Relative Expression of mRNAs in Human Epicardial, Substernal, Omental, Mesenteric, and Subcutaneous Adipose Tissues

Currently it is thought that it is the increases in visceral (intraperitoneal) rather than subcutaneous (extraperitoneal) adipose tissue is linked to the enhanced risk of diabetes, hypertension and cardiovascular disease in obesity [7–10]. Exactly how visceral adipose tissue is linked to this is unclear. It could be due to greater release of inflammatory factors by visceral fat or fatty acids and adipokines released by visceral adipose tissue that are preferentially delivered to the liver through the hepatic portal system.

The visceral fat is composed of omental and to a lesser extent mesenteric adipose tissue. The search for a major biochemical difference between these two types of visceral fat and abdominal subcutaneous fat of extremely obese women has turned up some interesting differences in gene expression (Table 4).

The gene expression of UCP-1, omentin, and haptoglobin in subcutaneous fat was less than 10% of that in omental fat. The data on UCP-1 confirm the initial report by Esterbauer et al. [107] that UCP-1 expression in subcutaneous fat was 12% of that in omental fat. However, the amount of UCP-1 gene expression, which is related to thermogenesis, in epicardial fat of humans is at least 9-fold greater than that in omental fat [106]. Sacks et al. [86] have postulated that the epicardial fat, which is located on the outside of the heart, serves to defend the myocardium against hypothermia.

Another protein whose gene expression was quite low (about 1%) in subcutaneous as compared to omental fat was omentin/intelectin (Table 4). It is also expressed at 3-fold higher levels in epicardial fat than in omental fat [108]. Its preferential expression in intraperitoneal adipose tissue probably reflects the fact that the blood vessels in these tissues arise from endothelial cells of the gut during development [108]. Unlike UCP-1, which is preferentially expressed in fat cells of omental fat (Table 2), omentin/intelectin is primarily found in the endothelial cells of the blood vessels [108].

It is unclear why haptoglobin is expressed at such low levels in subcutaneous fat but its expression is also low in mesenteric fat (Table 4). In contrast UCP-1 is found at the same level of expression in mesenteric fat as in omental fat while omentin/intelectin is found at far lower levels in mesenteric than in subcutaneous fat. As for the low level of expression of ATR_2 in subcutaneous fat that is probably due to overexpression of ATR_1 in subcutaneous fat.

Comparison of mesenteric with omental fat indicates that they have more in common with each other than with subcutaneous fat. This is especially true with regard to expression of UCP-1, prostaglandin D_2 synthase, angiotensinogen, ZAG, $NF\kappa B_1$, ATR_2, RBP-4, IL-6, and osteopontin.

However, MCP-1, IL-1β, adrenomedullin, PPARγ, and PAI-1 were expressed at significantly lower levels in mesenteric than in omental fat while their expression in subcutaneous fat was the same as or higher than that in omental fat. At this time these are simply lists of similarities and differences between omental and mesenteric fat indicating that they are different tissues. It is also not yet established whether the differences in mRNA expressionbetween omental and mesenteric fat are in the fat or the nonfat cells. Furthermore we know almost nothing about the physiological differences in the metabolism and adipokine release of these two kinds of intraperitoneal fat.

There have been many studies comparing the differences in response of isolated fat cells derived from omental as compared to subcutaneous fat and pieces of adipose tissue from these depots [109, 110]. However, the data are confusing since the results have been almost as varied as the number of reports. This is especially true for PAI-1 gene expression and protein release. Some reported greater in vitro release of PAI-1 by omental than by subcutaneous fat [10], others no difference in gene expression or protein content between omental and subcutaneous [111] while yet another group reported greater release by subcutaneous than omental adipose tissue from extremely obese humans [112]. This is a common occurrence in studies comparing omental versus subcutaneous fat of humans and it is unclear why such variable results are obtained.

The picture with regard to leptin gene expression and release is equally controversial. While some groups have reported greater expression and secretion by subcutaneous as compared to omental fat [113, 114] another group reported no difference [110] and a similar finding is in Table 4.

TNFα is one adipokine that is expressed (Table 4) and released to the same extent by human omental and subcutaneous adipose tissue [96, 115]. Another inflammatory adipokine is lL-6 that is released at higher levels by omental adipose tissue than by subcutaneous adipose tissue [62, 80] but the gene expression of IL-6 was higher in freshly isolated subcutaneous adipose tissue (Table 4).

Lipolysis is reported to be greater in adipoctyes derived from subcutaneous than from visceral adipose tissue and attributed to the greater size of the subcutaneous adipocyes [116]. However, similar levels of expression for hormone sensitive lipase (HSL) and perilipin have been reported in subcutaneous as compared to omental adipose tissue (Table 4, [117, 118]).

Giorgino et al. [109] have reviewed the evidence that fat cells isolated from omental fat are more insulin-responsive than those from subcutaneous human fat. Higher levels of insulin receptor expression have also been seen in omental as compared to subcutaneous adipose tissue [117, Table 4].

The visceral fat is composed of the intraperitoneal omental and mesenteric in the peritoneal cavity as well as

Table 4: Comparison of mRNAs in human mesenteric and subcutaneous as compared to omental adipose tissue from extremely obese women.

mRNA	subcutaneous as ratio of omental	mesenteric as ratio of omental
mRNAs lower in both subcutaneous and mesenteric as compared to omental		
Omentin/intelectin	0.01 ± 0.01***	0.14 ± 0.03***
Angiotensin II receptor 2 [ATR_2]	0.04 ± 0.01***	0.35 ± 0.10**
Haptoglobin	0.08 ± 0.01***	0.38 ± 0.10***
Nerve growth factor β [NGFβ]	0.22 ± 0.02***	0.44 ± 0.14**
Complement factor C3	0.31 ± 0.05***	0.47 ± 0.08***
VEGFR/FLT-1	0.41 ± 0.09***	0.66 ± 0.15*
PGC-1α	0.41 ± 0.14***	0.73 ± 0.06**
Insulin receptor	**0.47 ± 0.08*****	**0.44 ± 0.14***
SIRT1/sirtuin 1	0.47 ± 0.15**	0.44 ± 0.09***
Collagen VI α3	0.48 ± 0.16**	0.60 ± 0.11*
mRNAs lower or higher in subcutaneous but not in mesenteric as compared to omental		
Uncoupling protein 1 [UCP-1]	**0.07 ± 0.01*****	**1.23 ± 0.60**
Prostaglandin D_2 synthase	0.27 ± 0.05***	1.06 ± 0.12
Angiotensinogen	**0.33 ± 0.04*****	**0.87 ± 0.20**
Bone morphogenetic protein 7 [BMP-7]	0.35 ± 0.19**	1.02 ± 0.16
Zinc α2 glycoprotein [ZAG]	**0.44 ± 0.11*****	**0.83 ± 0.06**
NFκB1 [p50]	0.54 ± 0.15*	0.81 ± 0.19
Cytochrome C oxidase	**1.45 ± 0.13*****	**1.10 ± 0.13**
Angiotensin II receptor 1 [ATR_1]	1.62 ± 0.18***	0.93 ± 0.21
NAPDH oxidase [$p67^{phox}$]	1.90 ± 0.22***	1.10 ± 0.17
CD 14	2.30 ± 0.37***	0.85 ± 0.25
25-hydroxyvitamin D3 1α hydroxylase	3.00 ± 0.64**	0.90 ± 0.48
Retinol binding protein 4 [RBP-4]	**3.10 ± 0.26*****	**0.87 ± 0.20**
Interleukin 6 [IL-6]	3.50 ± 0.55***	0.66 ± 0.34
Osteopontin	4.90 ± 0.51***	1.07 ± 0.21
mRNAs lower or higher in mesenteric but not in subcutaneous as compared to omental		
Monocyte chemoattractant protein 1	1.15 ± 0.45	0.15 ± 0.04**
Interleukin 1β [IL-1β]	0.62 ± 0.30	0.20 ± 0.04***
Adrenomedullin	**0.97 ± 0.09**	**0.38 ± 0.13***
PPARγ	**1.23 ± 0.15**	**0.44 ± 0.11****
β1 adrenergic receptor	1.00 ± 0.07	1.74 ± 0.27*
mRNAs higher in subcutaneous and lower in mesenteric as compared to omental		
Plasminogen activator inhibitor 1	1.62 ± 0.27*	0.20 ± 0.04***
mRNAs higher in subcutaneous and mesenteric as compared to omental		
α1 glycoprotein	7.00 ± 0.86***	1.74 ± 0. 27**

mRNAs the same in subcutaneous and mesenteric as compared to omental (ratios were 0.50 to 1.50 of that in omental and not statistically significant).
ACE, **adiponectin, adipsin, amyloid A**, cathepsin S, caveolin-1, **CIDEA,** CD68, cyclophilin, endothelin-1, **FABP-4, FAT/CD36,** Giα2, **GPX-3,** heme oxygenase-1, HIF1α, **11β HSD-1, HSL,** IL-8, **leptin,** lipocalin-2**, LPL**, NADPH oxidase [$gp91^{phox}$], NGFβ [p65 RelA], eNOS, osteoprotegerin, **perilipin,** PRDM-16**,** TNFα, **Toll like receptor 4, UCP-2,** visfatin.
The values were obtained by qPCR as described in [106] and are expressed as the ratio ± sem of 5 to 15 paired comparisons from as many different individuals of the amount of mRNA in mesenteric and subcutaneous fat as compared to omental fat from the same woman. The mRNAs enriched in fat cells by at least 3-fold are shown in bold. Statistically significant differences are denoted as follows: $^*P \geq .05$, $^{**}P \geq .01$, and $^{***}P \geq .005$.

the intrathoracic fat depots of the substernal and epicardial fat. The latter two fat depots differ in that the epicardial surrounds the heart while the substernal fat body is a separate tissue within the thoracic cavity. Gene expression in substernal can be compared to that of epicardial fat to distinguish possible differences between these two intrathoracic depots. Fain et al. [106] found that of 45 mRNAs all except five were expressed in substernal fat at levels within 0.4 to 1.6-fold of that in epicardial fat. These were haptoglobin (21-fold greater), prostaglandin D_2 synthase (6-fold greater), nerve growth factor (5-fold greater), VEGFR/FLT1 (5-fold greater) and α1 glycoprotein (2-fold greater) with greater expression in epicardial as compared to substernal fat. UCP-1 is also expressed at in epicardial fat at 5-fold higher amounts than

in substernal fat [86]. Of these only UCP-1 is expressed at greater levels in fat cells than in the nonfat cells of human omental adipose tissue (Table 2). These data are compatible with the hypothesis that the fat cells in epicardial fat have a unique function as a brown fat-like tissue and could be involved in thermogenesis.

Epicardial fat has been postulated to be an inflammatory organ releasing adipokines that contributes to coronary artery disease because of the unique anatomical relationship between this fat and the coronary arteries [119]. However, when the gene expression of IL-6, IL-1β, PAI-1 or cyclooxygenase-2 was compared in epicardial fat of patients undergoing coronary artery bypass surgery to that in obese individuals undergoing gastric bypass surgery their expression in epicardial fat was less than 25% of that in omental fat [106]. It could be argued that this was because the bypass patients differed in other aspects, which they did, but the expression of 20 other protein ranged from 0.4 to 1.3 in omental fat to that seen in epicardial fat. In contrast, significantly higher amounts (1.6 to 2-fold greater) of the insulin receptor, ZAG, leptin, angiotensinogen and LPL were expressed in epicardial fat as compared to that in omental fat [106]. The significance of these differences between epicardial and omental fat remains unclear but do not suggest that epicardial fat is more inflamed than omental fat.

In conclusion, the reported differences in gene expression, hormonal sensitivity, and release of adipokines by visceral as compared to subcutaneous adipose tissue have been almost as varied as the number of reports [109, 110]. Furthermore, they provide few clues that can explain the putative harmful effect of enhanced accumulation of visceral fat. The fat cells found in visceral fat are smaller than those of subcutaneous fat from obese individuals but is that due to greater breakdown of large fat cells in visceral fat?

There are clear differences between mesenteric and omental fat but again it is unclear what they represent. Comparisons of visceral omental versus subcutaneous fat are probably influenced by the degree of obesity and this was demonstrated for PPARγ where the ratio in visceral to subcutaneous was around 0.2 at a body mass index of 20 but increased to about 1.2 in individuals with a body mass index of 50 [117]. Future studies will require the development of procedures to accurately assess the gene expression and release of adipokines by the different human adipose tissue depots under more physiological and reproducible conditions.

Recently the microRNA (miRNA) profiles of human omental and subcutaneous have been compared in humans without or with diabetes [120]. The expression of 155 miRNAs was examined and some differences were found that were said to correlate with fat cell phenotype, obesity, and glucose metabolism [120]. However, no miRNA was found exclusively in one fat depot versus the other suggesting a common developmental profile [120].

I conclude that the gene expression profile of omental fat clearly differs from that of subcutaneous fat for some proteins. However, none of these differences appear to explain the putative harmful effects of visceral obesity. Furthermore, there is scant agreement in the literature with respect to most proteins. This is possibly due to small sample sizes, sex differences, age differences, the extent of obesity, and the disease status of the humans from whom fat samples were obtained. For ethical reasons samples of omental and subcutaneous fat cannot be obtained from healthy donors. Most samples of human omental fat have been obtained from individuals undergoing gallstone, gynecological, or bariatric surgery. While individuals healthy enough to undergo bariatric surgery are extremely obese, the normal weight individuals always have some underlying disease process that could affect gene expression and adipokine release.

9. What Is the Link between TLR-4, Enlarged Fat Cells, and the Inflammatory Response Seen in Obese Humans

Recently the toll-like receptor 4 (TLR-4), that plays an important role in innate immunity through its ability to recognize bacterial lipopolysaccharides, has been postulated to play a role in the obesity-induced inflammatory response [95, 121, 122]. A loss-of-function mutation in TLR-4 prevents diet-induced obesity in mice and the development of insulin resistance [95, 121]. In macrophages and cultured adipocytes potent inducers of TLR-4 gene expression are bacterial lipopolysaccharides resulting in the release of inflammatory adipokines [123, 124]. In a monocyte/macrophage cell line (RAW 264.7) saturated, but not unsaturated fatty acids, induced the expression of COX-2 expression via TLR-4 [123]. Schaeffler et al. [122] reported that saturated fatty acids could induce the secretion of MCP-1 and other inflammatory adipokines in murine 3T3L1 adipokines through a pathway involving TLR-4.

Lin et al. [124] originally suggested that a fully intact pathway of innate immunity was present in rodent adipocytes that could be activated by bacterial lipopolysaccharides. Subsequently, functional TLR-4 has been found in human fat cells [125, 126] and the data in Table 2 indicates that in human omental fat the gene expression of TLR-4 is 5-fold greater in fat cells than in the nonfat cells. Zha et al. [127] reported that in vitro differentiated adipocytes had more TLR-4 mRNA than did preadipocytes and that TNFa secretion was induced by free fatty acids. My laboratory has similar findings in that the TLR-4 mRNA expression in human omental adipocytes differentiated in vitro was also 5-fold higher than that in preadipocytes (John N. Fain, unpublished experiments). In omental adipose tissue explants incubated for 48 hours TLR-4 gene expression was down regulated by about 70% but this was blocked in the presence of dexamethasone [128]. This may reflect a down-regulation of TLR-4 secondary to the 90 to 700-fold activation of the expression of inflammatory cytokines such as I-8, IL-6 and IL-1β that was markedly inhibited by dexamethasone [128].

It has been suggested that the hypertrophied fat cells seen in extreme obesity release large amounts of saturated fatty acids secondary to macrophage-induced lipolysis occurring in fat cells [129]. There is evidence in rodent

adipocytes that bacterial lipopolysaccharides can stimulate lipolysis via TLR-4 [130]. However, addition of bacterial lipopolysaccharides to explants of human adipose tissue incubated for 48 hours enhanced release of IL-1β, IL-6, and IL-8 by 50% to 70% under conditions where there was no significant increase in lipolysis (John N. Fain, unpublished experiments). Possibly breakdown of hypertrophied fat cells could be the primary trigger for the inflammatory response via activation of TLR-4 by fatty acids in neighboring intact fat cells resulting in the release of inflammatory adipokines that cause monocyte recruitment into the adipose tissue and insulin-resistance. However, this hypothesis is probably an over-simplification since thiazolidinediones appear to enhance the breakdown of large fat cells and the accumulation of small fat cells but this is associated with enhanced insulin sensitivity [105].

It was surprising to find TLR-4, whose function has traditionally been thought of as being involved in pathogen-associated molecular recognition by immune cells, expressed at higher levels in fat cells than in nonfat cells in human fat cells. The physiological function, if any, of this enhanced expression remains to be elucidated. Another unanswered question is what is the primary trigger that results in the accumulation of activated macrophages in the adipose tissue of extremely obese humans?

10. Hypoxia as the Primary Trigger of the Inflammatory Response

This hypothesis was originally proposed in 2004 by Trayhurn and Wood [1] and discussed in recent articles [131–134]. The best evidence for the "hypoxia hypothesis" is the evidence that adipose tissue is poorly oxygenated in the obese [134, 135]. The mechanisms involved are not understood beyond the accepted paradigm that HIF1α activation occurs resulting in activation of NFκB leading to increased gene transcription of inflammatory adipokines. Yin et al. [133] recently suggested that hypoxia in adipose tissue activates lipolysis and inhibits fatty acid uptake by adipocytes leading to activation of an inflammatory response via TLR-4. There is no evidence that activation of lipolysis per se induces an inflammatory response in human fat. Fain et al. [136] reported that growth hormone in the presence of dexamethasone, but not in its absence, stimulated lipolysis by explants of human omental adipose tissue over a 48 hours incubation but this was not accompanied by an increase in IL-8 gene expression or release.

Another problem is that while there is evidence that the adipose tissue from the ob/ob mouse is hypoxic in comparison to fat from obese mice, there was no increase in expression of VEGF while there was of hypoxia response genes such as HIF-1α, IL-6, Il-1β, and TNFα [134]. A similar finding has been reported by Halberg et al. [137] and remains to be explained since the current paradigm is that hypoxic tissues release VEGF that leads to increased tissue vascularization. However, the hypothesis may be incorrect or angiogenesis may also require other, as yet unknown, factors.

An attractive hypothesis is that as fat cells expand there is insufficient neovascularization to keep the cells from becoming hypoxic. This results in activation of HIF1α and a variety of responses including increased formation of inflammatory adipokines as well as activation of collagen synthesis and crosslinking of collagen involving lysyl oxidase [137]. There is global upregulation of extracellular matrix formation that hampers oxygen access to the cells and the increased stress resulting from expansion of the fat cells resulted in rupture of very large cells [137]. The fatty acids resulting from breakdown of triacylglycerols released by ruptured fat cells could activate macrophages as well as intact fat cells.

Alternatively, hypoxia leads to the death of large fat cells and macrophages are drawn to areas of recent cell death by mediators still to be described that are released after cell death, as suggested by Rausch et al. [135]. It may well be that visceral omental fat cells are more liable to lysis which explains why these fat cells are smaller than those found in subcutaneous adipose tissue. Furthermore it is commonly accepted, but may be an over-simplification, that visceral adipose tissue has more macrophages than subcutaneous adipose tissue and releases more inflammatory adipokines. Explants, but not isolated fat cells, of omental adipose tissue have been shown to release more PGE_2, PAI-1, IL-6, and VEGF than abdominal subcutaneous adipose tissue on a per g basis [62]. Similar results have been reported for IL-8 content of and release by visceral omental as compared to subcutaneous human adipose tissue [138].

11. Summary

The data in Figure 5 summarizes the relative release of selected adipokines by fat cells and nonfat cells of human adipose tissue. Of the adipokines shown in the figure only leptin, FABP-4, GPX-3, and adiponectin are expressed at 5 to 80-fold higher levels in fat cells than the other cells present in human fat and primarily released by fat cells. Adiponectin and GPX-3 are listed in blue because their circulating levels are lower in obesity.

The adipokines with black lettering are those whose circulating levels are enhanced in obesity and whose total release by adipose tissue explants is enhanced in obesity: IL-6, IL-10, ACE, TGFβ1, ICAM-1, TNFα, IL-1β, PAI-1, and IL-8 that are released by nonfat cells. However IL-10 may be an anti-inflammatory adipokine primarily released by the nonfat cells, whose circulating levels as well as in vitro release are elevated in obesity. The release of leptin and FABP-4 by fat cells is also enhanced in human obesity. It should be understood that most of these adipokines act locally and whether the changes in circulating levels of adipokines seen in obesity reflect release by adipose or other tissues remains to be established.

Omentin/intelectin is a novel adipokine preferentially found in visceral fat depots, especially human epicardial fat whose site of origin is the endothelial cells of blood vessels. For this reason it is listed in Figure 5 as being derived from the endothelial cells in the vessel wall. In conclusion, most of adipokines whose circulating levels are elevated in obesity and whose release by human adipose tissue is enhanced in

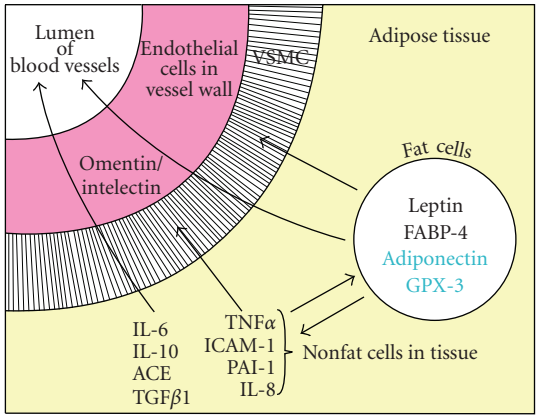


Figure 5: The relationship between adipokine release and paracrine signaling in human adipose tissue. The adipokines are divided into those released by fat cells [leptin, FABP-4, adiponectin, and GPX-3] and those by nonfat cells in adipose tissue [IL-6, IL-8, IL-10, ACE, PAI-1, ICAM-1, TNFα, TGFβ1, and omentin/intelectin]. Adipokines shown in black are those whose circulating levels are elevated in obesity as well as their release by incubated human adipose tissue explants. Circulating levels of adiponectin and GPX-3 are shown in blue since they are not elevated in obesity. Omentin/intelectin is shown as being secreted by the endothelial cells of the blood vessels of omental but not subcutaneous fat [108]. The arrows depict possible targets of the adipokines as the other cells in adipose tissue, as well as vascular smooth muscle cells (VSMC) and endothelial cells in the blood vessel walls plus release into the circulation (lumen of blood vessel).

obesity are inflammatory adipokines primarily derived from the nonfat cells of human adipose and other tissues.

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Review Article

Role of Heme Oxygenase in Inflammation, Insulin-Signalling, Diabetes and Obesity

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Diabetes and obesity are chronic conditions associated with elevated oxidative/inflammatory activities with a continuum of tissue insults leading to more severe cardiometabolic and renal complications including myocardial infarction and end-stage-renal damage. A common denominator of these chronic conditions is the enhanced the levels of cytokines like tumour necrosis factor-alpha (TNF-α), interleukin (IL-6), IL-1β and resistin, which in turn activates the c-Jun-N-terminal kinase (JNK) and NF-κB pathways, creating a vicious cycle that exacerbates insulin resistance, type-2 diabetes and related complications. Emerging evidence indicates that heme oxygenase (HO) inducers are endowed with potent anti-diabetic and insulin sensitizing effects besides their ability to suppress immune/inflammatory response. Importantly, the HO system abates inflammation through several mechanisms including the suppression of macrophage-infiltration and abrogation of oxidative/inflammatory transcription factors like NF-κB, JNK and activating protein-1. This review highlights the mechanisms by which the HO system potentiates insulin signalling, with particular emphasis on HO-mediated suppression of oxidative and inflammatory insults. The HO system could be explored in the search for novel remedies against cardiometabolic diseases and their complications.

1. Background

There has been a dramatic rise in the number of patients with the metabolic syndrome, a comorbid condition of hypertension, obesity, and diabetes. Diabetes mellitus is a chronic syndrome of impaired carbohydrate, protein, and fat metabolism caused by insufficient secretion of insulin and/or defects in insulin action in tissues due to insulin resistance. The incidence of diabetes is increasing globally [1] and type-2 diabetes (TD2) accounts for almost 90% of the cases diagnosed [2–4]. It is projected that the prevalence of T2D may reach 366 million in 2030 [1]. Similarly, the condition of obesity has escalated as more than 300 million adults, the majority of whom live in the developed world, are affected [5]. Obesity is amongst the main risk factor for insulin resistant T2D, hypertension, and other cardiovascular and renal complications [6]. Although inadequate insulin production is traditionally linked to type-1 diabetes (T1D), emerging evidence suggests that pancreatic beta-cell mass is reduced during the early stages of T2D and declines further with the progression of disease, eventually leading to loss of beta cells and reduced insulin production [7, 8]. This is consistent with previous observation indicating that T2D is not solely due to insulin resistance but also due to a failure of the insulin producing beta-cells to secrete an adequate amount of insulin [9]. On the other hand, in T1D it is a well-established concept that genetic defects trigger autoimmunity leading to the destruction of pancreatic beta cells and insulin insufficiency [10], and these events are further accentuated by apoptosis [11–13]. Similarly, in T2D, intense inflammatory activities characterized by the presence of cytokines, apoptotic cells, immune cell infiltration, amyloid deposits, and fibrosis may cause reduction of pancreatic beta-cell mass [14]. In both T1D and T2D, elevated inflammatory events play a major pathophysiological role in the disruption of islet architecture [10, 14–20]. Several factors are responsible for inflammation in T1D and T2D. These include dyslipidemia, hyperglycaemia, elevated nuclear-factor kappaB (NF-κB) activity, increased levels of adipokines such as tumour necrosis factor-alpha

(TNFα), interleukins (ILs), resistin, leptin and free fatty acids [14, 21]. Seen in this light, the suppression of apoptosis, necrosis, and intraislet inflammatory/immune events may be important for the preservation of islet architecture and beta-cell morphology. Therefore, the regulation of beta-cell number through the processes of proliferation, neogenesis, and apoptosis is important to safeguard islet function [22, 23] and the maintenance of adequate insulin production in T1D and T2D. Taken together, these studies suggest that impaired insulin secretion is not only an important etiological factor in the pathogenesis of T1D and T2D, but also an important pathophysiological driving force that is capable of dictating the dynamics and progression of the disease. Thus novel therapeutic modalities capable of suppressing inflammatory/immune responses, apoptosis, and necrosis would be beneficial in the conditions of T1D and T2D.

Generally, insulin resistance and T2D frequently occur in obesity [24–35]. Amongst the contributing factors, are overnutrition and inactivity. As an adaptive response to insulin resistance, pancreatic islets enhance their secretory activity. In most individuals, such an adaptation does occur during early stages of overnutrition and metabolism would appear normal at this stage. However, at later stages, this adaptation eventually fails in some individuals, depending on the genetic ability of the beta-cell to adapt and the severity of the resistance to insulin [36]. The reasons for this failure to maintain sufficient insulin secretion are a combined decrease in beta-cell mass and insufficient secretion of insulin. This reduction of insulin levels may be due to elevate inflammation, oxidative stress, amyloid deposition, lipotoxicity, and glucotoxicity [36]. Obesity and insulin resistance are associated with a state of low-grade inflammation due to chronic activation of innate immune system [37]. Although epidemiological studies have linked inflammation with obesity for decades, the underlying mechanisms remained obscured until the last decade. It is now widely accepted that the activation of inflammatory mediators such as NF-κB, TNFα, and c-Jun-N-terminal kinase (JNK) is amongst the common causes of insulin resistant T2D in obsessed conditions [24–35]. Thus, novel strategies that can preserve beta-cell integrity improve insulin sensitivity, and counteract inflammatory mediators like NF-κB, TNF-α, and JNK would be useful in the prevention and management of insulin resistant T2D and related cardiometabolic complications. Recent evidence has highlighted the important role of the heme oxygenase (HO) in insulin release and glucose metabolism [38–52]. Beside its emerging antidiabetic effects, the HO system is also known to abate oxidative stress and immune/inflammatory response [53–57]. This review will highlight the mechanisms by which the HO system potentiates insulin signalling, with particular emphasis on HO-mediated suppression of inflammation.

2. The HO System and Insulin Signaling

HO is a microsomal enzyme that cleaves the α-methene bridge of heme moiety to produce equimolar amounts of carbon monoxide (CO), bilirubin, and iron [58, 59] (Figure 1). CO and bilirubin are known to suppress apoptosis, necrosis, inflammation, and oxidative stress [56, 60–69], while the iron formed enhances the synthesis of the antioxidant, ferritin [70, 71].

The main isoforms of HO include HO-1 (inducible) and HO-2 (constitutive) isoforms [58, 59, 72, 73]. HO-1 and HO-2 are largely responsible for HO enzymatic activity [58, 72, 73], while the third isoform, HO-3, has no functional genes in rat and is considered a pseudotranscripts of HO-2 [74, 75]. The basal HO activity is maintained by HO-2 [58, 59, 72, 73, 76], while HO-1 is stimulated by a wide variety of different physical, chemical, and pathophysiological stimuli including oxidative and inflammatory insults [58, 59, 77–80], as well as metabolic and hemodynamic factors such as high glucose [80], elevated blood pressure [64], and lipids [81]. Therefore, HO-1 may be considered a sensitive index that is triggered in the onset of pathophysiological changes. However, in most cases the pathophysiological activation of HO-1 results only to a transient or marginal increase of HO-1 that falls below the threshold necessary to activate the downstream signalling components of the HO system [59, 63, 82]. For example, the pathophysiological activation of HO-1 by the hemodynamic stress of elevated blood pressure is not accompanied by changes of important component of HO-signalling like cyclic guanosine monophosphate (cGMP) [59, 63, 82–85]. Therefore the transient upregulation of HO-1 that normally accompanies many pathophysiological conditions may represent the first line of defense mounted against tissue injury to counteract adverse changes that would destabilize the homeostatic conditions in physiological milieu. Since the pathophysiological activation of HO-1 may fall below the threshold necessary to activate important signalling components through which the HO system elicits its effects of restoring tissue homeostasis [63, 82], a more robust enhancement of HO-1 would be needed to surmount the threshold [63, 82–85]. This can be achieved by pharmacological agents capable of inducing HO like some metalloprotoporphyrin such as hemin (ferric protoporphyrin IX chloride), stannous mesoporphyrin, copper protoporphyrin, and cobalt protoporphyrin. Given that many of the adverse factors which stimulate HO-1 such as elevated blood pressure [64] and high glucose and lipid [80, 81] concentrations are implicated in the pathophysiology of metabolic syndrome, the HO system may constitute a novel approach that could be explored against metabolic syndrome and related cardiometabolic complications (Figure 2).

The emerging role of the HO system in insulin release and glucose metabolism is becoming increasingly clear [38–52]. HO-mediated stimulation of insulin release has been reported in different rats strains [38, 46, 49–52] and mice [86, 87]. These studies suggest a central role of CO in glucose metabolism. In the human body, CO is formed at a rate of 16.4 μmol/h and daily production of may reach 500 μmole [88]. Interestingly, under normal physiological conditions, islets of Langerhans produce CO and nitric oxide (NO) to regulate insulin release [45, 46]. While NO negatively modulates glucose-stimulated insulin release, CO stimulates insulin secretion [45, 46]. Moreover, glucose stimulates

pancreatic beta-cells to produce CO, which in turn triggers insulin release [45, 46]. The critical role of the HO system in insulin release and glucose metabolism was reported in Goto-Kakizaki (GK) rats, a model with defective pancreatic beta-cell HO-2 [38]. Since HO-2 is largely responsible for basal HO activity [58, 59, 72, 73, 76] and thus the production CO, the impairment of the HO system in GK rats resulted in reduced CO and insulin insufficiency [38]. Interestingly, treatment with the HO-inducer, hemin, or CO corrected the defective HO system and enhanced insulin release with improvement of glucose metabolism [38]. Collectively, these studies suggest that reduced beta-cell CO and/or impaired HO system may lead to dysfunctional glucose metabolism.

3. The Role of HO System in Inflammation and Insulin Resistance

The inflammatory and metabolic systems are among the most fundamental for survival, and these systems have been evolutionarily well-conserved in species [37]. However, the conditions of nutrient-overload or obesity may offset these systems leading to inflammation in metabolic sites like the adipose tissue, liver, and skeletal muscles. One consequence of such imbalance is the increased production of proinflammatory cytokines, adipokines, and other inflammatory/oxidative transcription factors including NF-κB activating protein (AP-1) and JNK. Although both JNK and NF-κB play important roles in inflammation-induced insulin resistance, accumulated evidence suggests that they do so through different mechanisms. The principal mechanism by which JNK causes insulin resistance is through the phosphorylation of serine residues in insulin receptor substrate-1 (IRS-1) [89–91]. However, since JNK is a stress kinase that also phosphorylates the c-Jun component of the AP-1 [92], the activation of AP-1 by JNK may contribute to aggravate inflammatory insults and hence insulin resistance. NF-κB causes insulin resistance by stimulating proinflammatory cytokines like TNF-α, IL-6, IL-1β, and resistin, which in turn activates JNK and NF-κB pathways to create a vicious cycle that will exacerbate tissue damage [89, 91, 93–97].

An important trigger of NF-κB, AP-1, and JNK is the renin-angiotensin-aldosterone system (RAS). Like angiotensin-II, aldosterone stimulates inflammation and fibrosis by activating transcription factors such as NF-κB, AP-1, and JNK [98, 99]. Moreover, oxidative stress will further enhance the activation of JNK [100]. On the other hand, JNK blocks insulin biosynthesis [100] and regulates AP-1 [101]. These transcription factors modify insulin signaling and thus are involved in the development of insulin resistance. Therefore, the reduction of oxidative/inflammatory transcription factors in T2D would not only limit tissue insults but also decrease the oxidative destruction of a wide variety of important metabolic regulators including adiponectin and insulin [100, 102]. Therefore, novel therapeutic strategies that concomitantly ablate inflammation and insulin resistanc, but enhance adiponectin are needed. Interestingly, the HO system has been shown to modulate both the metabolic and inflammatory systems suppressing insulin resistance and inflammation while enhancing adiponectin levels [40–44, 47, 48, 51, 55, 56, 82, 103–113]. Therefore the inflammatory and metabolic effects of HO may be highly integrated and the proper function of each may depend on the other [37]. Given that insulin resistance may trigger inflammatory events [114], it remains to be clarified whether insulin resistance precedes the development of inflammation or vice versa. Further investigation in this regard will advance our knowledge in the development of more specific therapeutic modalities.

Adiponectin is a cytoprotective protein produced by the adipose tissue. It is composed of several multimeric species or isoforms with low-, middle-, or high-molecular weights [115]. The high-molecular-weight isoform is thought to be the most clinically relevant. Generally adiponectin elicits its effects through its receptors (adiponectin receptor-1 and -2) which, besides activating adenosine monophosphate protein kinase (AMPK), also activates peroxisome proliferator-activated receptor alpha (PPARα) in the liver to increase insulin sensitivity and decrease inflammation [116–118]. Generally, the high-molecular weight adiponectin plays a crucial role in obesity-linked insulin resistance and metabolic syndrome. Interestingly, PPARγ upregulates high-molecular weight adiponectin to enhance insulin sensitivity and glucose metabolism [117, 119, 120]. Besides its insulin-sensitizing effect, adionectin has also protective effects against atherosclerosis [121] and inflammation [122]. Moreover, clinical evidence indicates that adiponectin levels are low in patients with obesity, atherosclerosis, and insulin resistance [119]. Furthermore, knocking-out adiponectin leads to insulin-resistant T2D [120]. Collectively, these studies underscore the important role of adiponectin in cytoprotection, insulin sensitivity, and glucose metabolism. Insulin insensitivity is a hallmark of T2D [123, 124] the causes include excessive NF-κB activity [125–129], elevated JNK activation [100] and increased production of adipokines including free fatty acids, TNFα, ILs, resistin, leptin by the adipose tissue [130–133]. In T2D diabetic patients, insulin resistance may lead to metabolic syndrome, a pathological condition with hyperinsulinemia, hypertension, glucose intolerance, and dyslipidemia [122, 134, 135].

We recently showed that the HO inducer hemin is endowed with potent antihypertensive and antidiabetic effects. Interestingly hemin therapy is effective against T1D and T2D. Our findings showed that upregulating the HO system with hemin reduced fasting and postprandial hyperglycaemia in different insulin-resistant T2D models, including nonobese Goto-Kakizaki rats (GK) [42, 44] and Zucker diabetic fatty rats (ZDF) [43], a genetically obese leptin receptor-deficient (fa/fa) model [136, 137]. Interestingly, after termination of therapy, the antidiabetic effects prevailed for 3 and 4 months, respectively, in GK and ZDF [42–44]. Further revelations from our findings indicate that hemin therapy is also effective against streptozotocin-(STZ-) induced diabetes [41] and improves insulin sensitivity/glucose metabolism in spontaneously hypertensive rats (SHRs) [47], a model of essential hypertension and with features of metabolic syndrome like insulin resistance and impaired glucose metabolism [138, 139]. Furthermore we showed that hemin improved insulin-signaling/glucose

metabolism in deoxycorticosterone-acetate (DOCA) hypertension, a model of primary aldosteronism [48], suggesting a role of the HO system against dysfunctional glucose metabolism in aldosteronism. Interestingly, the antidiabetic effect of hemin was accompanied by a paradoxical increase of plasma insulin and enhanced insulin-sensitivity [41–44], alongside the potentiation of agents that promote insulin-signalling, including adiponectin [40–44, 47, 48, 108–113] cGMP [45, 140], cyclic adenosine monophosphate (cAMP) [45], adenosine monophosphate-activated protein-kinase (AMPK) [141, 142], aldolase-B [143], and glucose-transporter-4 (GLUT4) expression [142, 144]. Correspondingly, hemin improved intraperitoneal glucose-tolerance (IPGTT), reduced insulin-tolerance (IPITT), and lowered insulin resistance (HOMA index), and the inability of insulin to enhance GLUT4 was overturned [41–44]. Furthermore, hemin therapy potentiated the antioxidant status in ZDF, GK, and STZ-diabetic rats with the suppression of oxidative/inflammatory mediators including 8-isoprostane, NF-κB, AP-1, AP-2, and JNK [41–44].

Given that diabetes is characterized by elevated oxidative and inflammatory insults, the HO system would suppress these insults by generating CO, bilirubin/biliverdin and ferritin against apoptosis, inflammation and oxidative stress [66–68, 71, 145–147]. Thus, the insulin-sensitizing effects of hemin, when combined to its antihypertensive effects [58, 59, 63–65, 83–85, 148–154], underscores the important role of the HO system that could be explored against impaired glucose metabolism and hypertension given the rising incidence of comorbidities of essential hypertension, glucose intolerance, and insulin resistance [155, 156] as well as pathophysiological conditions like primary aldosteronism, glucose intolerance, and insulin resistance [157–159].

3.1. The HO System, NF-κB, and Inflammation. The HO-1 promoter harbours consensus binding sites for many substances including inflammatory/oxidative transcription factors like NF-κB, AP-1, and AP-2 as well as motifs for glucocorticoid-responsive elements [160, 161]. As such, the HO system may regulate inflammation and insulin release [41–44, 47, 48, 162]. Given that HO-1 is induced by different stimuli including high glucose levels [77, 80], the diversity of HO inducers may be indicative of multiple regulatory elements for the HO-1 gene with binding sites for different transcription factors or genes. These arrays of genes may account for the diverse and pleitropic effects of the HO system in many cellular events including defence and glucose metabolism [40–44, 47, 48, 65, 163–166]. By modulating a wide variety of transcription factors, cellular metabolism may be regulated. Thus, the HO system may be crucial for the coordination and proper functioning of basic physiological units in animals. More importantly, the regulation of NF-κB by HO-1 may be important for cellular homeostasis given the pleitropic effects of NF-κB-signalling in many pathophysiological conditions including inflammation and insulin resistance [125–129] (Figure 2).

Transcription factors are proteins that act as a sensor to monitor cellular change and convert the signals into gene expression. Generally, a specific cellular signal pathway can activate multiple transcription factors and the expression of a specific gene may be controlled by multiple transcription factors. Importantly, transcription factors mediate signal transduction by binding to specific DNA sequence in gene promoters to regulate transcription activity. Although the exact characterization of the series of events and the mechanisms that integrate the inflammatory response with metabolic homeostasis at the cellular and systemic level are not fully understood, emerging data indicates that NF-κB plays a key role [125, 127, 128, 167–169]. NF-κB is a family of transcription factors that generally function as heterodimers to regulate specific gene expression. In the quiescent state, NF-κB is trapped in the cytoplasm by its interaction with the inhibitory protein, "inhibitor of NF-κB kinase subunit beta" (IKKβ). The IKKβ/NF-κB complex is an essential mediator of inflammatory cascades. Importantly, the IKKβ/NF-κB complex plays a critical and fundamental role for immunity and survival [125, 167]. The proteosomal degradation of the IKKβ/NF-κB complex is triggered by different stimuli or pathophysiological conditions. Upon activation by stimuli like oxidative stress, lipopolysaccharide endotoxin (LPS), mitogens, or cytokines, the phosphorylation of Ser177 and Ser181 activates the complex, triggering a cascade of reactions that leads to proteolysis of IKKβ-specific protein kinase and the release of the NF-κB. Upon release, NF-κB translocates into the cell nucleus where it mediates the transcriptional activity of a wide variety of target genes [170–172]. The transcriptional products of NF-κB in immune cells include different cytokines and their receptors, adhesion molecules, immune modulators, and apoptotic factors, all of which are implicated at various stages during the inflammatory cascade.

Besides its traditional role in the immune/inflammation system, emerging evidence suggests that NF-κB also mediates chronic low-grade metabolic inflammation in a variety of different tissues including adipose [128], liver [168], and skeletal muscle [127, 169]. Therefore NF-κB can interfere with several molecular programs to cause the different aspects of metabolic dysfunction, especially under chronic conditions like hypertension, diabetes, and obesity or nutritional excess. For example, the NF-κB has been linked to insulin resistance and numerous physiological deregulations that underlie overnutrition [125–129]. Generally, insulin resistant T2D is associated with the chronic activation of NF-κB pathway and elevated inflammation [126, 173, 174].

A commonly used strategy to alleviate tissue insults and restore cellular metabolism in conditions of elevated inflammation and insulin resistance is PPARγ agonists [175]. PPARγ agonists are a class of drugs used against insulin resistance and T2D [175]. PPARγ is a genetic sensor of fatty acids and a member of the nuclear receptor superfamily of ligand-dependent transcription factors. PPARγ is required for fat cell development and is the molecular target of thiazolidines, a class of insulin-sensitizing drugs that exert their effects in adipose tissue and skeletal muscle [175]. Although a variety of PPARγ agonists are available [175], novel pharmacological agents would be needed in the therapeutic armament giving the recent escalation of insulin

resistant T2D, metabolic syndrome, and cardiometabolic complications.

We recently showed that upregulating the HO system with hemin suppressed NF-κB in different models of T2D including ZDF and GK rats [42–44], as well as different hypertensive models including SHR [47, 84] and DOCA-hypertensive rats [48, 65, 84, 150, 151]. Similarly, other HO inducers has been shown to be effective against insulin resistant T2D [39, 40, 46, 49, 111, 113, 176]. Therefore, HO inducers may be explored in the design of novel strategies against insulin resistant diabetes. Incidentally, PPARγ have been shown to upregulate high-molecular weight adiponectin [117, 119, 120], an insulin-sensitizing agent. Similarly, adiponectin is upregulated by the HO system [40–44, 47, 48, 108–113]. Therefore the synergistic effects of PPARγ and the HO system in improving insulin sensitivity and glucose metabolism may be a novel approach to combat insulin resistance and related cardiometabolic complications.

3.2. The HO System, cJNK, and Inflammation. JNK proteins belong to the mitogen activated protein kinase family and control transcriptional activity of AP-1 via phosphorylation of c-Jun [92]. Three closely related JNK isoforms including JNK1, JNK2, and JNK3 have been described. Generally, JNK-signalling is activated by inflammatory cytokines and environmental stressors [177]. Reports indicate that the different JNK isoforms are implicated in a wide variety of pathophysiological conditions caused by inflammatory insults. These include insulin resistance, T2D, infectious diseases, stroke, Parkinson's disease, and cardiovascular disorders [92]. The tissue distribution and activities of JNK1, JNK2 and JNK3 isoforms are different. JNK1 and JNK2, are widely expressed in tissues and are involved in different activities including T-cell activation and brain development [92]. On the contrary, JNK3 is less-diffused and is predominantly expressed in neurons in the hippocampus and mediates neuronal apoptosis.

In obesity, JNK activity is increased in the liver, muscle, and fat tissues probably due to the increase of free fatty acids and TNF-α [92, 177]. Interestingly, JNKs are key signalling molecules that link inflammation and insulin resistance (Figure 2). The role of JNK in insulin resistance is highlighted in studies showing that the abrogation of JNK prevents insulin resistance in obese and diabetic mice [178–180]. In contrast, overexpression of a dominant-negative proteins for JNKs or knocking down JNK1 by RNA interference assay resulted in the inhibition of JNK with improved insulin sensitivity [178–180]. Similarly, genetic disruption of JNK1 gene reportedly prevented the development of insulin resistance in obese and diabetic mice [181]. Moreover, under diabetic conditions, oxidative stress activates JNK, which in turn suppresses insulin biosynthesis [100] causing impaired insulin-signalling and glucose metabolism. Conversely, the suppression of JNK resulted in reduced insulin resistance and improved glucose tolerance in diabetic mice [100].

The role of JNK in insulin resistance has been further highlighted by its interaction with IRS-1. An important step during the insulin-signal transduction cascade is the activation of insulin receptor tyrosine kinase and the resulting phosphorylation of IRS-1. Subsequently, through the activation of phosphatidylinositol 3-phosphate kinase (PI3K), insulin regulates different metabolic pathways. These include the activation of glucose uptake in muscle and fat, downregulation of gluconeogenesis in liver, upregulation of glycogen synthesis, and induction of protein synthesis. However, these important insulin-mediated signalling events could be halted if serine of the IRS-1 is phosphorylated instead of tyrosine. Several stress-related kinases, including JNK, induce the serine phosphorylation of IRS-1 and thus inhibit the insulin-signal transduction cascade. Interestingly, JNK-mediated phosphorylation of serine is a common pathophysiological event in obesity [90, 91]. In a related study, obesity-induced stress was shown to cause insulin resistance via JNK-mediated phosphorylation of inhibitory serine residues IRS-1 [90, 91]. Collectively, these studies underscore the important role of JNK in insulin resistance and suggest that inhibitors of JNK-signalling may be used as insulin sensitizing agents. Thus, the genetic ablation of one or more JNK isoforms may be a novel strategy against insulin resistant T2D and related obesity-induced cardiometabolic complications.

A number of different pharmacological agents capable of inhibiting JNK are presently under investigations. These include different classes of inhibitors: small-molecule JNK inhibitors which may be derivatives of anthrapyrazolone, imidazoles, anilinoindazole, pyrazoloquinolinones, aminopyridines, or pyridine carboxamide [182, 183]. Other classes of compounds under studies are ATP-competitive JNK inhibitors and peptide substrate-competitive ATP-noncompetitive JNK inhibitors [182, 183]. These include diaryl-imidazoles, anilinoindazoles, pyazoloquinolinones, aminopyridines, pyridine carboxamides, anilino-bipyridines, and anilino-pyrimidines and compound SP600125 [182, 183]. Although these compounds are promising as they are endowed with good potency and greater selectivity, their practical application in clinics is a long way ahead; so other alternative modalities to block JNK-signalling would be useful. Interestingly, we recently showed that upregulation of the HO system with hemin suppressed JNK and improved insulin sensitivity and glucose metabolism in STZ-induced diabetes, insulin resistant T2D models like ZDF and GK; as well as in hypertensive models like SHR and uinnephrectomised DOCA-salt rats [41–44, 47, 48]. The attenuation of JNK by hemin was consistent with previous reports in which an upregulated HO system reportedly abrogated JNK [184]. Although significant contributions have been made in delineating the role of JNK and its isoforms in cardiometabolic complications, further studies are needed to identify more specific inhibitors and/or novel compounds with improved pharmacokinetics and pharmacodynamics.

3.3. The HO System and Obesity and Inflammation. Obesity and insulin resistance are pathophysiological cardinal features of metabolic syndrome. Generally, obesity and insulin

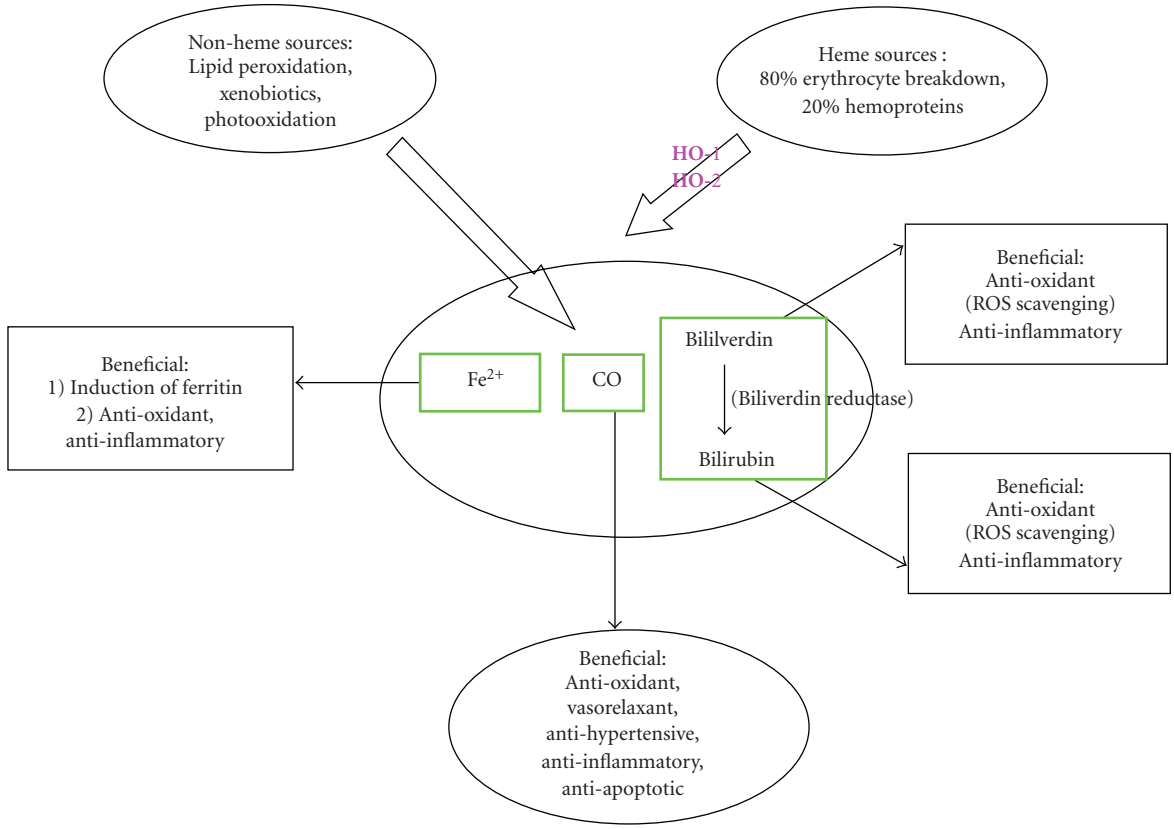

Figure 1: In the human body, carbon monoxide is formed at a rate of 16.4 μmol/h and daily production can reach 500 μM (Piantadosi, *Antioxid Redox Signal,* 2002, 4:259-70). About 86% comes from HO-catalyzed degradation of heme while 14% from lopid peroxidation xenobiotics and other sources.

resistance are closely associated with a state of low-grade inflammation of white adipose tissue as a result of chronic activation of the innate immune system leading to impaired glucose tolerance, diabetes and other cadiometabolic complications [37]. Although epidemiological studies had linked inflammation with obesity for decades, the underlying mechanisms remained obscured until the last decade when strong evidence indicated that obesity is a condition associated with chronic inflammatory activity due to incessant activation of a wide variety of inflammatory mediators including NF-κB, TNF-α and JNK [25–35]. Similarly, free fatty acids binding innate immune receptors like Toll-like receptor (TLR4) have been shown to trigger significant inflammatory activities in the condition of obesity. Consistent with this notion are reports indicating that in TLR4-knockout mice, diet-induced obesity and inflammation is abrogated [185]. On the other hand, the binding of free fatty acids to TLR4 activates the IKKβ/NF-κB complex and the JNK pathway to initiate a cascade of other inflammatory and proinflammatory factors [186]. Therefore, the secretion of proinflammatory factors by the adipose tissue and the regulation of these secretions by increasing adiposity sustain the notion of an ongoing low-grade inflammatory process in obesity. Emerging evidence indicates that adipocytes from different body compartments have distinct inflammatory phenotype based on their anatomical location and genetic differences between intraabdominal visceral-fat and peripheral subcutaneous-fat [187]. Importantly, visceral adiposity is more malignant than subcutaneous adiposity. These differences are reflected in the contrasting roles of visceral and subcutaneous adiposity in the pathogenesis of obesity-related cardiometabolic complications like insulin resistant T2D and coronary artery disease in lean and obese individuals [187]. Generally, resident macrophages in visceral adipose tissue generate higher levels of proinflamatory cytokines like TNF-α and IL6, but reduced levels of the anti-inflammatory adipokine, adiponectin [187]. Changes in the levels of these cytokines are amongst the fundamental causes of inducing insulin resistance and play a major role in the pathogenesis of endothelial dysfunction, T2D, and related cardiometabolic complications like atherosclerosis, especially in the condition of obesity.

In the adipose tissue chronic overnutrition leads to macrophage infiltration, resulting in local inflammation that potentiates insulin resistance. Both TNF-α and JNK are implicated in inflammation-induced impairment of insulin signalling in obesity [25–31]. Moreover, NF-κB is a stimulator of TNFα [91, 93–97]. The role of NF-κB in inflammation in obesity was demonstrated experimentally in metabolic tissue, by nutrient overload [32, 33]. Accordingly, glucose overload was shown to activate NF-κB in the adipose [128], endothelial, and pancreatic tissues [188–190]. Similarly, lipid overload increased NF-κB activity in humans and animals [128, 191]. Moreover, in cultured

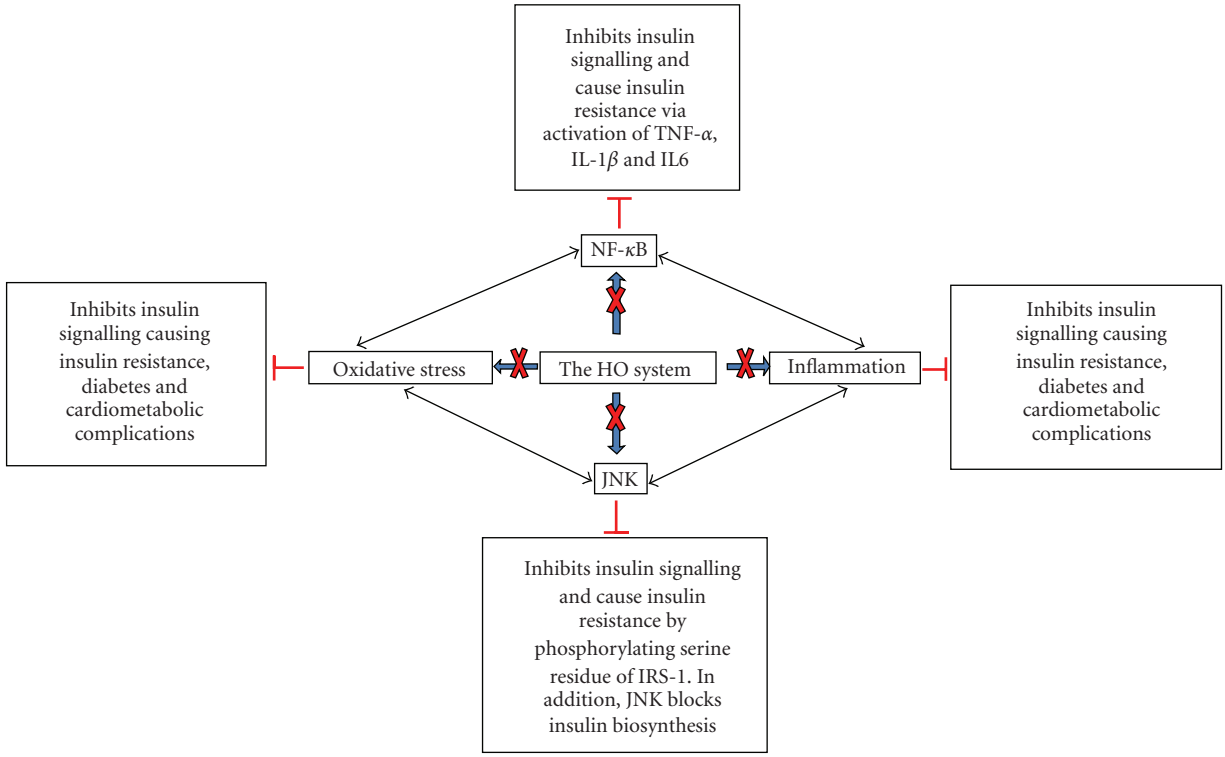

Figure 2: Schematic representation illustrating the protective role of the HO system in glucose metabolism. Inflammatory and oxidative mediators like NF-κB, JNK, TGF-α, IL1β and IL-6 are amongst the pathophysiological factors that impair insulin signalling. Generally these substances stimulate oxidative/inflammatory events destroying tissue. Conversely, other factors including cytokines and inflammatory/oxidative transcription factors like NF-κB, JNK stimulate a variety of different pathophysiological pathways to further aggravate oxidative/inflammatory insult, creating a vicious cycle of intense inflammation that would severely damage tissue and compromise many physiological functions including glucose metabolism. However, the HO system suppresses these inflammatory/oxidative mediators and pro-inflammatory cytokines to enhance insulin signalling and improve glucose metabolism.

cells, tissues and whole animals, NF-κB has been shown to activate TNFα, IL6, IL-1β, and plasminogen activator inhibitor 1 (PAI-1) inducing insulin resistance [91, 93–97]. Collectively, these studies strongly suggest a role of the NF-κB pathway in nutrition-overload induced insulin resistance and its involvement in aggravating inflammation and exacerbating insulin resistance. Moreover, the presence of NF-κB in different tissues may trigger distinct signals to mediate the complex manifestations of overnutrition-induced diseases. Therefore the activation of the NF-κB may be considered not only a key mechanism for the development of insulin resistance but also an important contributor for metabolic dysfunction and the development of nutrition-overload induced complications. Seen in this light, blockade of NF-κB activity would be imperative to maintain cellular homeostasis and adequate physiological function in obesity (Figure 2). Moreover, dysfunctional metabolism due to excessive inflammation may lead to premature aging in obesity.

Although obesity is escalating in all population groups, a causal relationship between obesity and premature aging has been postulated for years. The molecular mechanisms involved in obesity-induced aging are only beginning to be unraveled now. Recent evidence suggests that obesity accelerates the aging of adipose tissue due to increased formation of reactive oxygen species in fat cells and shortened telomeres which ultimately results in activation of the p53 tumor suppressor, inflammation, and the promotion of insulin resistance and hypertension [192, 193]. Therefore obesity may be considered a chronic stress factor that creates a pathphysiological milieu that may ultimately compromise the metabolic system. Overnutrition-induced chronic stress offsets the balance between metabolic and immune functions and contributes to the development of visceral obesity, T2D; and the metabolic syndrome. Moreover, obesity-induced proinflammatory cytokines from the adipose tissue may act as an additional chronic stimulus for stimulation of other stress-related pathways including the hypothalamic-pituitary-adrenal axis [194], creating a vicious cycle between metabolic and immune responses during nutrient overload. Accordingly, obesity-induced stress has been reported to impair the systemic metabolic homeostasis [37]. Conversely, stress has been linked to the development of visceral obesity [177]. Generally, stress is characterized by elevated levels of glucocorticoid, a hormone implicated in the development and differentiation of preadipocytes [195]. Reports indicate that glucocorticoids regulate the expression of the stress-related enzyme 11b-hydroxysteroid dehydrogenase (11b-HSD). This enzyme has dual function as it converts inactive cortisone to active 11b-HSD1 or, alternatively, the conversion

of cortisol to inactive 11b-HSD2 [196]. 11b-HSD1 induces stress and has been linked to the development of obesity and insulin resistance [197–199]. Supportive of this notion are experiments demonstrating that knocking-out 11b-HSD1 suppressed the development of obesity and insulin resistance, whereas overexpression of 11b-HSD1 led to the development of obesity [197–199]. Consistently, the activity of 11b-HSD is elevated in obsessed humans [200, 201]. Of more-importance and even more intriguing is the finding that the ability to regulate 11b-HSD is lost in T2D patients, whereas it is compromised in nondiabetic obsessed individuals [201]. These findings highlight the central role of glucocorticoids in regulating metabolism via 11b-HSD, and suggest that the regulation of 11b-HSD is a dynamic process that becomes gradually impaired or even completely compromised as the severity of the obesity worsens when it progresses to metabolic syndrome and/or T2D. Interestingly motifs for glucocorticoid-responsive element are present in the HO-1 promoter [160, 161]. Whether this is indicative of a role of the HO system in the modulation of glucocorticoid-induced stress and/or involvement in glucocorticoid-induced regulation of 11b-HSD remains the subject of future investigations. However, this hypothesis is particularly attractive because stress is linked to the development of visceral obesity [177], a condition in which glucocorticoids play a key role in the development and differentiation of preadipocytes [195]. Interestingly, the HO system has been shown to suppress visceral and subcutaneous obesity [40, 111–113, 202]. Therefore, the HO-mediated suppression of visceral and subcutaneous obesity when combined to other cyto-protective effects of the HO system such as the attenuation of NF-κB activity [41–44, 47, 83, 84, 203] may constitute a protective shield against insulin resistance, obesity, and other nutrition-overload related complication (Figure 2). Accordingly, the presence of motifs for glucocorticoid-responsive elements and binding sites for many substances including sites for inflammatory/oxidative transcription factors like NF-κB, AP-1 and AP-2 in the HO-1 promoter [160, 161] suggest that the HO system may be playing a more important role in metabolism that previously thought.

Although obesity was first described as low-grade inflammation more than a decade ago, it is only recently that obesity-induced increase of macrophage infiltration of adipose tissue and elevated number of classically activated macrophages or M1-type has been associated with obsessed individuals [204–206]. It is becoming increasingly clear that the adipose tissue is infiltrated by macrophages that trigger inflammatory events in obesity [207, 208]. Moreover, the dramatic shift of the pool of macrophages from the alternatively-activated M2-type to the classically-activated M1-type results in changes in secreted cytokines from predominantly anti-inflammatory (M2-type) to proinflammatory (M1-type) in obese conditions, although the exact mechanism for this shift remains largely unclear [204–206]. Since alternatively activated macrophages have a beneficial role in regulating nutrient homeostasis, an increase of alternatively-activated M2-type might be a useful strategy for treating insulin resistant T2D [205]. Given that PPARγ is necessary for the maturation of alternatively activated macrophages [205], and PPARγ is a transcription factor that regulates adipogenesis, insulin sensitization and inflammation, the potentiation of PPARγ-signalling would be beneficial in obesity [209–213].

Interestingly emerging evidence indicates that the HO system suppresses different inflammatory events including macrophage infiltration [54, 63, 111, 202, 214] and potentiate insulin sensitivity and glucose metabolism in obesity [40, 111, 113] in a similar way as PPARγ [209–213]. Accordingly, cross-talk between PPARγ and the HO system has been reported [215]. Moreover, analysis of human HO-1 promoter using a combination of transient transfection experiments, mutational analysis, and gel shift assays has demonstrated the direct transcriptional regulation of HO-1 by PPARγ and PPARα [215]. Consistently, the notion that HO-1 is a PPAR target gene [216, 217] has been further strengthened by the observation that HO-1 enhances the levels of PPARγ protein expression and activity [218]. On the other hand, PPARγ has also been shown to induce HO-1 [217]. Therefore, a mutual reciprocal stimulatory relationship between PPARγ and the HO system can be envisioned [217, 218] and coordination of this synergistic interaction between these two systems may constitute a novel and potent strategy to combat obesity-induced complications and other related problems like T2D, insulin resistance, hypertension, and metabolic syndrome. Given the recent findings that HO inducers enhance insulin sensitivity and improve glucose metabolism in different insulin resistant rats strains including ZDF and GK [219, 220] and obese mouse [40, 111, 113], it is tempting to speculate that the HO-mediated suppression of macrophage infiltration constitutes not only an important anti-inflammatory mechanism to limit tissue insult in hypertension but also a mechanism that could be explored to improve insulin sensitivity and glucose metabolism in obsessed individuals with insulin resistance and overt T2D.

3.4. The HO System, Oxidative Stress, and Insulin Signalling. Many studies have underscored the role of oxidative stress in insulin resistance [174, 221–223]. Reactive oxygen species are produced by the electron transport system in mitochondrial respiration and are increased in conditions associated with enhanced oxidation of energy substrate such as glucose and free-fatty acids. Reports indicate that factors that increase oxidative stress like hyperglycemia, free-fatty acids and adipokines contribute to insulin resistance [174, 222]. Although the exact mechanism of insulin resistance is not fully understood, recent data suggest the implication of oxidative stress in the pathogenesis of multiple forms of insulin resistance [174, 221–223]. Thus, there is a general consensus that elevated oxidative stress unleash the cascade of events that impairs insulin-signalling [174, 222, 223]. As such, insulin resistance may be regarded as a state of increased exposure to reactive oxygen species [174, 222], and thus strategies that concomitantly reduce oxidative stress, glucose/insulin intolerance and lower blood pressure may improve glucose metabolism. Generally, the skeletal muscles accounts for 65%–90% of the clearance of glucose

clearance [140]. Under healthy conditions, the vascular actions of insulin stimulate the production of NO from the endothelium leading to vasodilation and increased blood flow to skeletal muscles that enhance glucose-uptake [224]. However, in hypertensive conditions, elevated levels of superoxide quenche NO by forming peroxynitrite [225], that subsequently oxidizes arachidonic acid to generate 8-isoprostane, a potent vasoconstrictor which may decrease skeletal muscle blood flow, and thus reduce glucose-uptake.

Although many studies support the link between hypertension and insulin resistance, the underlying mechanisms are not completely understood. However, CO from the HO system and NO may be implicated because these vasoactive gases are important not only as a vasodilators, but also in the regulation of insulin signaling [45, 46, 226–230]. Recent evidence indicates that insulin stimulates the production of NO [45, 46, 226], and thus insulin may regulate blood pressure via the NO pathway. The binding and subsequent activation of IRS-1 and IRS-2 by insulin triggers a cascade of events that ultimately lead to activation of PI3K and protein kinase (PKB) or Akt. In healthy subjects, both P13K and Akt activate endothelial NO synthase to generate NO [231, 232] and thus promote vasodilation. However, in insulin-resistant conditions, oxidative stress impairs the activation of P13K/Akt-signaling resulting in impaired vasorelaxation [232–234]. Similarly, TNFα impairs vasorelaxation by inhibiting the P13K/Akt-signaling [233, 235]. The P13K/Akt-signaling is important for glucose transport and is involved in the translocation of GLUT4 to the cell membrane [232]. However, in hypertensive subjects, these cascades of events may be impaired, and so insulin-stimulated NO may be insufficient [232] leading to reduced vasorelaxation, decreased blood to skeletal muscles, and impaired translocation of GLUT4. Thus, hypertension and insulin resistance may compromise endothelial function and cause overt T2D.

Since GLUT4 and effective dilation of skeletal muscle and are largely responsible for glucose disposal, reduced GLUT4 translocation and impaired skeletal muscle dilation would result in reduced removal of glucose, leading to hyperglycemia, hyperinsulinemia, and eventually insulin resistance [232, 236]. Alternatively, diminished action of insulin and the resultant hyperglycemia may result in the accumulation of advanced glycation end-products (AGE) and this would increase oxidative/inflammatory events [237–239], which in turn would further increase the production of AGE, and thus creating a vicious cycle that potentiates the oxidative destruction of beta-cells in both T1D and T2D [237, 240–242]. Moreover, increased oxidative stress and AGE may lead to DNA damage, the activation of NF-κB, and deranged transcription [235], all of which will accentuate cell damage. Therefore the progressive loss of beta-cell function and the corresponding decline of insulin production reported in TD1 and TD2 could be attributed, at least in part to oxidative stress [243, 244]. Accordingly, the maintenance of specialized islet architecture and the regulation of beta-cell number by antioxidants and antiapoptotic agents may be important for the preservation of intact pancreatic structure to safeguard the insulin-producing capability of beta-cells. Interestingly, our recent studies indicate that upregulating the HO system enhances GLUT4 expression and improves glucose metabolism [41–44, 47, 48]. On the other hand, the P13K/Akt-signaling may also regulate vascular contractility and blood pressure homeostasis by modulating calcium ion transport [232, 234, 245]. Moreover, insulin triggers vasodilatation by inhibiting voltage-gated calcium influx [232, 234]. Similarly, glucose transport and glucose-6-phosphate synthesis have been reported to reduce smooth muscle vascular resistance by enhancing calcium efflux [232, 234]. The P13K/Akt-signaling and glucose transport may be blunted in the pathophysiological conditions like insulin resistance and hypertension [232, 234]. The dysfunctional P13K/Akt-signaling coupled to reduced calcium efflux may result in elevated vascular resistance in insulin resistant diabetes and hypertensive conditions [232, 234]. Therefore oxidative stress, impaired glucose transport and utilization, and reduced NO production are amongst the contributing factors of hypertension and these factors may also lead to the development of insulin resistance [232, 233, 246].

From the above mentioned studies, it could be envisaged that elevated vascular resistance may constitute a common denominator in hypertension and insulin resistant diabetes, and strategies like HO inducers that enhance vascular relaxation [228, 229] and improves glucose metabolism [38–52] may constitute an alternative approach to simultaneously combat hypertension and insulin resistance in patients symptomatic with these comorbid conditions. However, given that many insulin resistant patients are normotensive, further studies are needed to fully characterize the P13K/Akt-signaling and calcium efflux in hypertension and insulin resistance. Given the close association between the P13K/Akt-signaling and the HO system [247–251], further exploration of these pathways may lead to better understanding of the multifaceted interaction between the HO system and the P13K/Akt-signalling and the development of novel strategies against hypertension and insulin resistance.

4. Concluding Remarks

Obesity, insulin resistant T2D, and many related cardiometabolic complications share a metabolic milieu characterized by elevated inflammatory/oxidative insults. While inflammation-induced insulin resistance is increasing in parallel with the epidemic of obesity and metabolic syndrome, there are additional unrelated mechanisms associated with the polygenic conditions of insulin resistance, T2D, and cardiometabolic complications that create a great challenge for future therapeutic modalities. With the polygenic nature of these conditions, treatment strategies should not be limited to monogenic targets. Interestingly, emerging data have underscored the role of the HO system in insulin sensitivity and cellular metabolism. The HO system has been shown to suppress visceral and subcutaneous obesity [40, 111–113, 202], potentiating the antioxidant status in cells and abating oxidative/inflammatory mediators including 8-isoprostane JNK AP-1 and AP-2 [41–44, 47, 83, 84, 203]. These qualities,

in combination to the HO-mediated attenuation of NF-κB activity [41–44, 47, 83, 84, 203] may constitute a protective shield against insulin resistance, obesity, and other nutrition-overload-related complications. Moreover, the presence of motifs for glucocorticoid-responsive elements and binding sites for many substances including sites for inflammatory/oxidative transcription factors like NF-κB, AP-1 and AP-2 in the HO-1 promoter [160, 161], suggest that the HO system may be playing a more important role in the regulation of cellular metabolism.

Finally, the mutual reciprocal stimulatory relationship between PPARγ and the HO system may be explored in the design of novel remedies. The coordination of this synergistic interaction may constitute a novel approach that could be explored in the search of more-effective and potent strategies against the polygenic conditions of insulin resistance, T2D, and cardiometabolic complications.

Abbreviations

(AP-1): Activating protein-1
(AP-2): Activating protein-2
(AMPK): Adenosine monophosphate-activated protein kinase
(AGE): Advanced glycation end-products
(CO): Carbon monoxide
(cAMP): Cyclic adenosine monophosphate
(cGMP): Cyclic guanosine monophosphate
(JNK): c-Jun-N-terminal kinase
(DNA): Deoxyribonucleic acid
(DOCA): Deoxycorticosterone-acetate
(GK): Goto-Kakizaki rats
(GLUT4): Glucose-transporter-4
(IKKβ): Inhibitor of nuclear factor kappa B kinase subunit beta
(IRS-1): Insulin receptor substrate-1
(IL-6): Interleukin
(IL-1β): Interleukin-1 beta
(IPGTT): Intraperitoneal glucose-tolerance
(IPITT): Intraperitoneal insulin-tolerance
(HO): Heme oxygenase
(HOMA): Homeostasis model of insulin resistance
(11b-HSD): 11b-hydroxysteroid dehydrogenase
(LPS): Lipopolysaccharide endotoxin
(NO): Nitric oxide
(NF-κB): Nuclear-factor kappaB
(PPARγ): Peroxisome proliferator-activated receptors gamma
(PKB or Akt): Protein kinase
(PI3K): Phosphatidylinositol 3-phosphate kinase
(RAS): Renin-angiotensin-aldosterone system
(RNA): Ribonucleic acid
(STZ): Streptozotocin
(TLR4): Toll-like receptor
(T1D): Type-1 diabetes
(T2D): Type-2 diabetes
(TNF-α): Tumour-necrosis factor-alpha
(ZDF): Zucker diabetic fatty rats.

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Research Article

The Effect of Chronic Candesartan Therapy on the Metabolic Profile and Renal Tissue Cytokine Levels in the Obese Zucker Rat

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The effect of candesartan, an angiotensin-II type-1 receptor antagonist, on the metabolic profile and renal inflammation is unclear. We evaluated this relationship by feeding male lean (LZ) and obese (OZ) Zucker rats chow or chow with candesartan (23.5 mg/kg · diet) for 14 weeks (n = 6–8/treatment/body type). Candesartan reduced serum triglycerides, plasma creatinine, urine albumin, and renal cortical collagen and glycogen deposition in the OZ. An ELISA-based cytokine array revealed that candesartan normalized elevated renal interleukin (IL) 1-β and monocyte chemoattractant protein-1 (MCP-1) levels in OZ. Nonetheless, candesartan impaired glucose tolerance, and did not lower blood insulin or glucose levels. Moreover, renal IL-1α, -2, -4, -6 and -10 tumor necrosis factor-α, interferon-γ, were significantly reduced in OZ relative to LZ, and increased by candesartan. Furthermore, candesartan increased growth-regulated oncogene, transforming growth factor-β1 and IL-18 in OZ kidneys to a level higher than LZ or untreated OZ. Candesartan did not affect renal cytokine levels in LZ. Overall, candesartan attenuated renal disease and improved renal function in OZ, despite mixed effects on metabolic factors and cytokines. Reduced plasma triglycerides and/or renal MCP-1 and IL-1β may have had a role in this protection. However, these effects were clearly independent of any improvement in glucose tolerance.

1. Introduction

Renal disease or nephropathy is a frequent complication of the metabolic syndrome and a leading cause of end-stage renal failure in type II diabetes [1]. Hypertension, inflammation, insulin resistance, and/or an altered metabolic profile, including poor glycemic control and dyslipidemia are among the several mechanisms or "risk factors" associated with this disorder [2–9]. Elucidation of these mechanism(s) is crucial in guiding the development of more efficacious therapies to combat renal disease, improve the quality of life in this patient population, and assuage the societal burden of the metabolic syndrome.

Angiotensin II (Ang II), a potent vasoconstrictor, and mediator of oxidative stress and proliferative pathways in tissue, is a strong candidate in modulating many of these risk factors [10–12]. Ang II raises blood pressure [13], induces insulin resistance [14, 15], and increases inflammation by either directly activating immune cells or by producing inflammatory mediators [16, 17]. Furthermore, clinical as well as basic research studies have shown that treatment with any of several available Ang II receptor blocker (ARBs), compounds that bind antagonistically to the Ang II, type I receptor (AT1R), results in renoprotection [18–21]. Candesartan (CAN), one such ARB, commonly prescribed to lower blood pressure (BP), has clearly been shown to improve renal function and attenuate renal disease [20, 21]; however the mechanism(s) underlying this protection is not entirely clear.

The obese Zucker rat is a model for human metabolic syndrome with associated renal disease [22, 23]. One manner in which CAN would be expected to exert significant renoprotection is by lowering BP. We have already published BP in response to chronic CAN therapy in the same

set of rats [24]. CAN treatment for 14 weeks resulted in a marked and sustained fall in BP of approximately 20–30 mm Hg, in both lean and obese rats [24]. ARBs have also been demonstrated to reduce inflammation in tissues, such as the pancreas, heart, brain, vasculature, and adrenal gland [25–27], improve insulin sensitivity [28–30], and activate PPAR-γ, an intracellular nuclear hormone receptor involved in the regulation of carbohydrate and lipid metabolism [31]. Therefore, the beneficial actions of ARBs on the kidney may extend well beyond their BP-lowering actions.

In this report, we examine the effects of chronic CAN therapy on renal function and disease in obese and lean Zucker rats. We determine whether attenuation of renal inflammation and/or factors associated with the metabolic syndrome may contribute to any observed renoprotective effects of CAN. We utilize a cytokine array to measure 14 cytokines/chemokines in the whole kidney homogenates of treated rats. In addition, we measure certain indices of the metabolic profile including plasma triglycerides and glucose tolerance. We hypothesize that chronic CAN therapy will reduce renal inflammation (perhaps via improvement in the metabolic status), in the obese Zucker rat, thus potentially contributing to attenuation of renal disease in these rats.

2. Methods

2.1. Animals Study Design. Thirty-two male Zucker rats (16 lean and 16 obese) were obtained from Charles River Laboratories (Wilmington, MA). Rats were singly housed in microfilter top, plastic cages with a normal 12-hour light/dark cycle according to protocols approved by the Georgetown Animal Care and Use Committee, an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care, International) approved facility. At about 9 weeks of age, 8 rats from each body type were randomly assigned to either ground control diet (Purina 5001 Rodent Chow, Purina Mills, St. Louis, MO) solidified in agar with 70% water, or the same base diet with 23.5 mg candesartan cilexetil (Atacand, AstraZeneca Pharmaceuticals, Wilmington, DE) incorporated per kg diet (wet weight). This resulted in an approximate dose of 3-4 mg/kg · bw/day of CAN in the treated rats. Rats were weighed weekly and fed diets and received water *ad libitum* for 14 weeks. Urine was collected at 12 weeks in metabolic cages.

2.2. Glucose-Tolerance Test. A glucose tolerance test (GTT) was given to all rats at 13 weeks to assess their ability to rapidly regulate blood glucose (a function of insulin sensitivity). The test was performed as described previously [32, 33]. Briefly, rats were given a 50% dextrose solution (3 ml/kg · bw) intraperitoneally. Glucose was measured in tail blood with a glucometer (One-Touch, Lifescan, Johnson & Johnson) after pricking the tail at 15, 30, 60, 90, and 120 minutes postglucose administration. Blood glucose concentration over time was plotted and the areas under the curves were calculated and statistically compared.

2.3. Kidneys and Blood Collection. At the end of the 14 weeks, rats were deeply anesthetized with sodium pentobarbital and the right kidney perfusion fixed, as described previously [34, 35], for histochemical analyses. Prior to perfusion, some blood was collected into both K_3-EDTA- and Na^+-heparin-containing vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). Immediately after euthanization, the left kidney was removed and processed as a whole kidney homogenate for the analysis of protein levels.

2.4. Plasma and Urine Analyses. Plasma insulin levels were analyzed in blood collected at euthanization by a radioimmunoassay, as previously described [36]. Triglycerides and urine albumin were analyzed by colorimetric assays (Sigma, St. Louis, MO and Exocell, Philadelphia, PA, resp.). Plasma creatinine was determined by the Jaffe rate method (Creatinine Analyzer 2, Beckman Diagnostics Systems Group, Brea, CA).

2.5. Histochemical Staining. After fixation with 4% paraformaldehyde, the right kidney was processed to paraffin, sectioned at 4 μm, and stained with periodic acid-Schiff's (PAS; for demonstration of glycogen deposition) to determine glomerulosclerosis, which is defined as thickening of the basement membrane and mesangial expansion) or Masson's trichrome (for demonstration of collagen deposition) to determine tubulointerstitial fibrosis, which is defined as tubular atrophy or dilatation, deposition of extracellular matrix, and interstitial fibroblast proliferation [37].

2.6. Cytokine Profile Using an ELISA-Based Cytokine Array. Levels of monocyte chemoattractant protein-1 (MCP-1), interleukins (IL)-1β, -1α, -2, -6, -5, -4, -10, -18, -12p70, tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), growth-regulated oncogene (GRO-KC), and granulocyte macrophage colony-stimulating-factor (GM-CSF) were determined in the whole kidney homogenates using a rat cytokine/chemokine LINCOplex premixed 96-well plate assay (Millipore, St. Charles, MO, catalog no. RCYTO-80K-PMX).

2.7. Western Blotting. Western blotting was performed as previously described [32] on whole kidney homogenates to evaluate the effects of body type and therapy on endothelial nitric oxide synthase (eNOS, NOS3) and TGF-β1 (transforming growth factor using commercially available antibodies: polyclonal NB100-91995 (TGF-β1, Novus Biologicals, Littleton, CO), and monoclonal 610297 (eNOS, Transduction Laboratories, San Diego, CA).

2.8. Statistics. To determine the overall effects of CAN treatment and body type on variables of interest, data were analyzed by two-way (body type $\times$ treatment) analysis of variance (ANOVA). The difference between individual pairs of means was analyzed by unpaired t-test. If data were nonparametric, not normally distributed, or variances were different, we used the Mann-Whitney rank sum test (Sigma Stat, Chicago, IL). $P < .05$ was considered significant for

all analyses. Among the 14 cytokines analyzed in the array, 6, that is, IL-1α, IL-4, IL-10, IL-12p70, IFN-γ and TNF-α, were below detection levels in the untreated obese group, as well as in some treated obese rats (1 or 2 out of 6). Thus the statistical analyses for these 6 cytokines were done in a different way; that is, cytokine levels were categorized (one category was "undetectable"), and a t-test (Rank Test) on categorical assignments was done. The other 8 cytokines were analyzed by standard unpaired t-test as continuous variables.

3. Results

3.1. Chronic Candesartan Treatment Improves the Indices of Renal Function and Reduces Pathology. Obese Zucker rats had significantly higher levels of plasma creatinine (Figure 1(a)) and urine albumin excretion (Figure 1(b)), relative to lean age-mates, indicating impaired renal function and advancing renal disease. Both plasma creatinine and urine albumin were markedly reduced in the obese rats treated with CAN. CAN also resulted in a significant reduction in these two parameters in the lean rats, although the reduction was of considerably lower magnitude.

In addition to improving renal function, chronic CAN therapy attenuated renal pathology in obese rats as revealed by histochemical staining. Untreated obese rats demonstrated marked renal pathology as indicated by features of glomerulosclerosis and tubulointerstitial fibrosis in their kidney sections relative to lean rats (Figure 2). Chronic CAN attenuated these pathological features. As shown in Figure 2, Masson trichrome-stained paraffin sections revealed heavy deposition of collagen in the interstitial spaces (light-blue staining, Figure 2(a) and enlarged lumens of the renal tubules in untreated obese rats only. Similarly, mesangial expansion (arrows) and hyaline casts in the renal tubules were found only in untreated obese rats' kidney by periodic Schiff's staining (Figure 2(b)). There was no apparent effect of the chronic CAN therapy on renal histology in the lean rats.

3.2. Candesartan Has an Opposite Effect in Lean and Obese Rats with Regard to Water Intake and Urine Volume. As expected, obese rats gained significantly more weight by the end of the study (Table 1). CAN treatment did not significantly affect weight gain, although there was a trend for weight gain to be less in the obese CAN rats. Absolute water intake and urine volumes were higher in obese rats; however, when normalized by kidney weight, urine volume was lower in obese control rats and corrected (to lean levels) by CAN. In contrast, CAN significantly decreased urine volume in lean rats, so that there was a significant interaction between terms in the 2-way ANOVA. There was also a strong trend for decreased water intake in these treated lean rats ($P = .064$, unpaired t-test between Lean Control and Lean CAN).

3.3. Candesartan Reduced Serum Triglyceride Levels. Serum triglycerides were significantly increased in obese rats related to lean (Table 1). Candesartan treatment significantly reduced triglyceride levels in obese rats (to a level only 46% of the untreated obese level), and to more modest extent in the lean (to 75% of the untreated lean level). Levels in treated obese rats were still on average 85% higher than lean control.

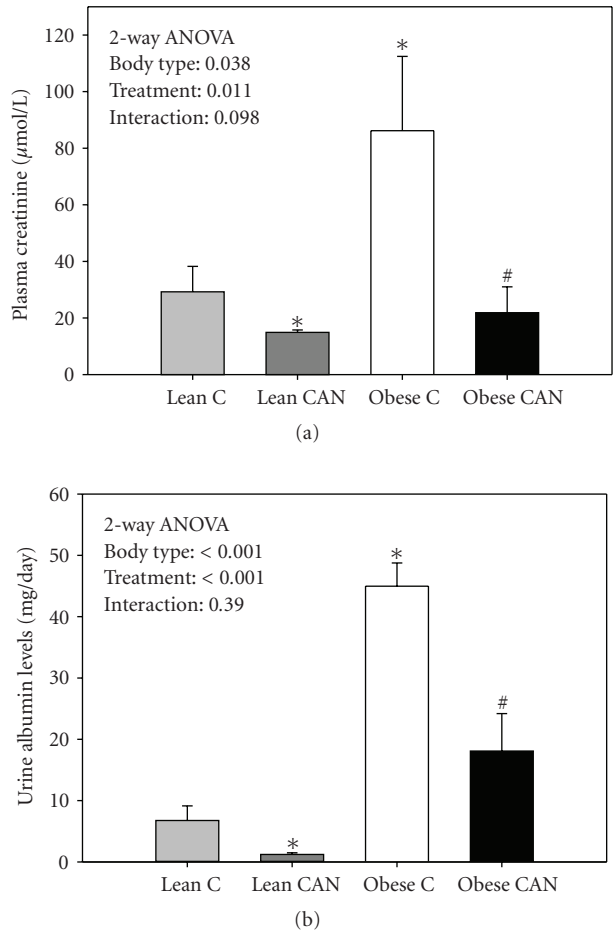
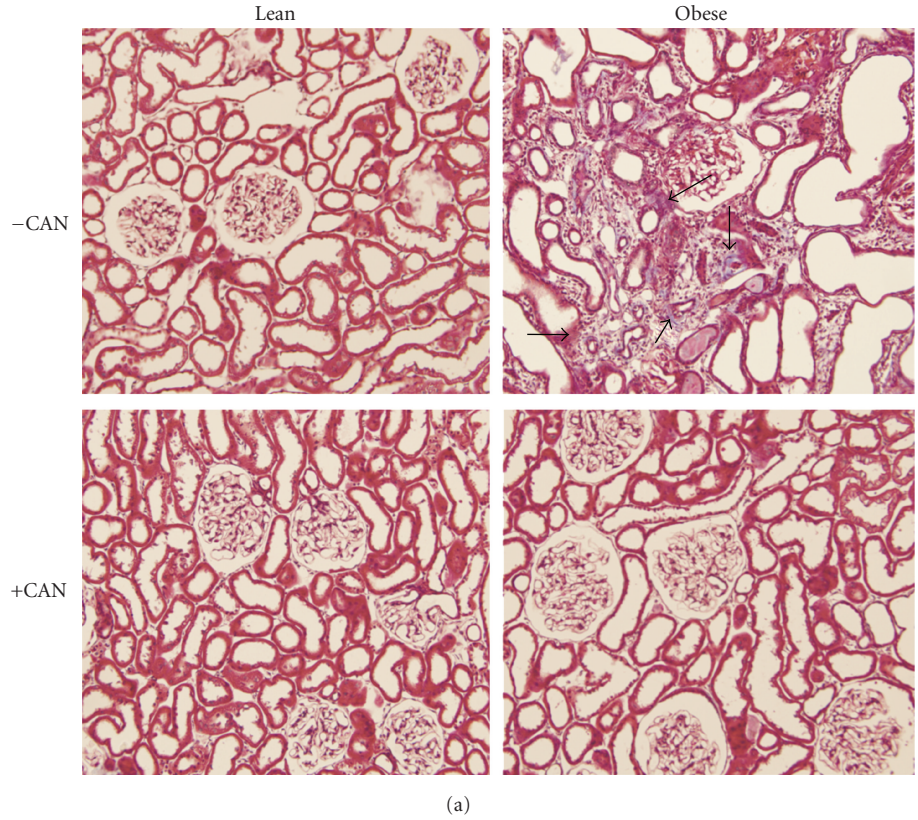
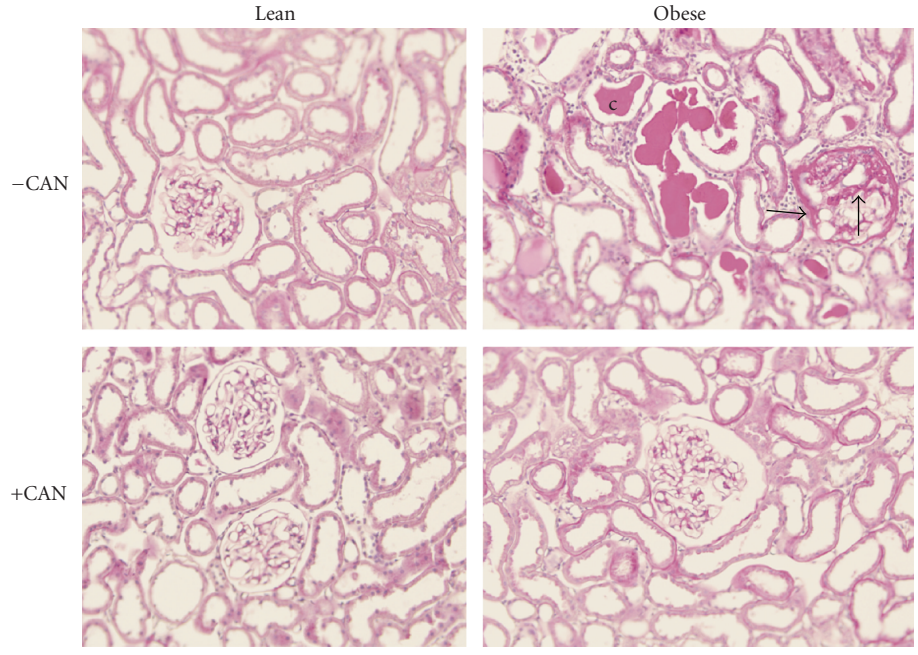


Figure 1: Plasma creatinine (a) and urinary albumin excretion (b) in candesartan-treated (CAN) or -untreated (c) lean and obese rats at the end of 14 weeks of treatment ($n = 8$ per body type/treatment). Obese had significantly increased albumin excretion and higher plasma creatinine relative to lean groups; CANtreated groups were significantly different from control by 2-way ANOVA ($P < .05$). $*$ indicates a significant difference ($P < .05$) from lean control mean and # from obese control mean by unpaired t-test.

3.4. Candesartan Has an Opposite Effect in Lean and Obese Rats with Regard to Glucose Tolerance. Obese rats demonstrated significantly slower plasma glucose clearance (glucose tolerance, measured at 13 weeks), in response to intraperitoneally administered glucose (3 ml/kg·bw), relative to lean rats (Figure 3(a)). Unexpectedly, CAN treatment further worsened this impairment, as demonstrated by significantly higher area under the curve (AUC) for plasma glucose levels over time in CAN-treated obese rats relative to untreated obese. In lean rats, however, CAN significantly improved glucose tolerance. This led to a significant interaction between body type and treatment by 2-way ANOVA.



(a)



(b)

Figure 2: Renal cortical pathology: glomerulosclerosis and tubulointerstitial fibrosis were assessed in periodic acid Schiff and Masson's trichrome-stained paraffin sections (4 μm sections, 6 sections/kidney were analyzed) from Zucker rats (Lean or obese) treated with (+CAN) or without candesartan (−CAN) (n = 3/bodytype/treatment). (a) Masson trichrome-stained paraffin sections showing enlarged lumens of the renal tubules and heavy deposition of collagen in the interstitial spaces (light-blue staining, arrows) in untreated rats only. (b) Periodic acid Schiff's-stained paraffin sections. The mesangial expansion is shown by arrows and hyaline casts by (c) in the renal tubules in untreated rats only.

Table 1: General physiology[†].

Treatment	Weight gain (g/14 weeks)	24-hour water intake (ml/d)	24-hour urine volume (ml/d)	24-hour urine volume (ml/g kidney weight/d)	Serum triglycerides (mg/ml)
Lean Control	124 ± 5	61 ± 3	48 ± 1	116 ± 4	1.44 ± 0.56
Lean CAN	119 ± 1	55 ± 1	42 ± 2*	104 ± 6	1.08 ± 0.07
Obese Control	232 ± 14*	73 ± 3*	51 ± 2	75 ± 3*	5.74 ± 0.95*
Obese CAN	191 ± 20*	107 ± 17*#	69 ± 7*#	107 ± 14#	2.66 ± 0.41#
Factors	Results of 2-way ANOVA for above parameters (*P*-values)				
Body Type	**<.001**	**<.001**	**<.001**	**.028**	**<.001**
Treatment	.065	.074	.174	**.024**	**.013**
Interaction	.145	**.016**	**.006**	**<.001**	**.044**

[†]mean ± sem, $n = 7$ or 8/group; *indicates a significant difference from lean control; #indicates a significant difference between obese control and obese CAN groups, by unpaired *t*-test. **In bold**- *P*-values < .05 by 2-way ANOVA (significant).

In addition, final blood glucose levels (measured just prior to euthanization at 14 weeks) trended toward being higher in obese rats treated with CAN relative to all other groups (Figure 3(b)), *P*-value = .07, as compared to lean control). Plasma insulin levels (Figure 3(c)) were significantly higher in the obese rats, and not altered by CAN in either lean or obese.

3.5. Effects of Long-Term Candesartan Treatment on the Cytokine Profile in Kidney Tissue. Renal levels of only two cytokines (out of 14) were elevated in obese versus lean rats in the control state, that is, MCP-1 and IL-1β (Figures 4(a) and 4(b)).Their levels were, respectively. 200% and 70% higher than untreated lean controls. Long-term CAN treatment reduced these levels in obese rats such that they were no longer significantly different than lean. There was no significant effect of CAN on the level of these two cytokines in the lean rats.

Surprisingly, the kidney levels of 9 out of the 14 cytokines were significantly lower in the untreated obese Zucker rats relative to lean controls. These were IFN-γ, IL-4, IL-2, IL-6, GM-CSF, IL-1α, IL-10, IL-12p70, and TNF-α (Figures 4 and 5). Some of these data are depicted as individual rat values (rather than means) because the protein was "below the level of detection" in some animals (Figure 5). Long-term CAN treatment increased the level of these proteins, such that they were no longer significantly different from lean control levels (except for IFN-γ, Figure 5(a),which remained significantly lower in CAN-treated obese rats). Similar to what was observed for MCP-1 and IL-1β, CAN had no effects on the levels of these 9 cytokines in the lean rats.

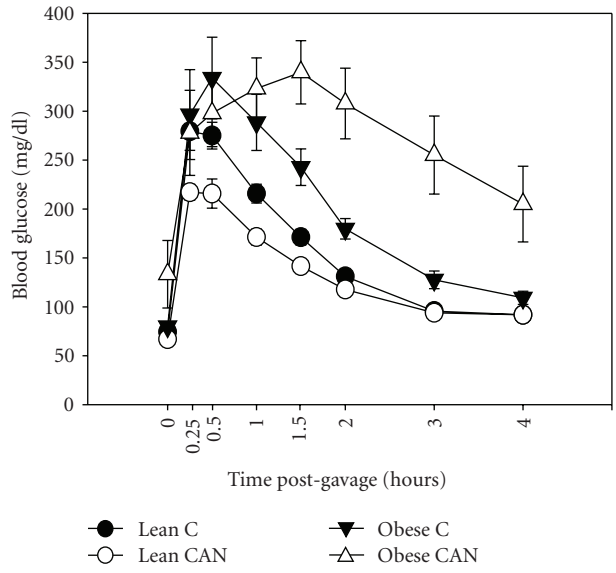
Finally, a different pattern emerged for renal levels of IL-18 and GRO-KC (Figure 4). These 2 cytokines were not significantly different between lean and obese rats, but increased by CAN treatment in obese rats only, so that the levels were elevated (relative to obese control rats). No significant differences were observed for IL-5 levels between any of the groups.

3.6. Effects of Candesartan Therapy on eNOS and TGF-β. In Figure 6, we show renal expression of endothelial nitric oxide synthase (eNOS), a protein central in oxidative-stress related pathways due to its generation of nitric oxide [38] and transforming growth factor β, a protein central in increased matrix formation and deposition in diabetic nephropathy [39]. Both eNOS and TGF-β were expressed at greatest levels in obese CAN-treated rats. Moreover, there was a significant interactive term for both proteins in that CAN reduced expression in lean rats but increased it in the obese.

4. Discussion

We have demonstrated that chronic candesartan treatment attenuated renal pathology and reduced renal levels of MCP-1 and IL-1β in obese rats. Consistently, it markedly improved renal function and lowered serum triglyceride levels in these rats. Unexpectedly, glucose tolerance was worsened. Moreover, renal levels of 11 out of 14 cytokine analyzed were in fact significantly increased by CAN in the obese rats. IL-18 and GRO-KC levels were highest in CAN-treated obese rats as compared to all other groups. Overall, our results from the cytokine array suggest that the regulation of renal cytokine levels by chronic candesartan-treatment of obese Zucker rats appeared to be primarily driven by normalization of kidney function and architecture and perhaps preservation of these necessary and sometimes protective inflammatory pathways. Decreases in serum triglyceride and/or renal MCP-1 and IL-β levels may have a role in the reno-protective actions of candesartan in the metabolic syndrome.

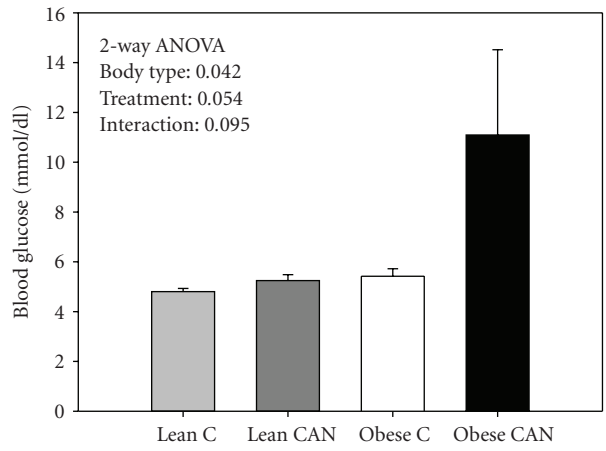
In many models of diabetes and insulin resistance, elevated RAS activity has been shown to be intimately intertwined with activation of inflammatory pathways in renal tissue [40]. In contrast to expectations, examining whole kidney homogenates, we found an increase in only two cytokines, IL-1β and MCP-1 in the obese Zucker rat kidneys, relative to lean controls. Increased mRNA expression of TNF-α, IL-1 and IL-6 has been reported in the kidney of streptozotocin-induced type 1 diabetic rats [41], a model associated with much higher levels of plasma glucose and no obesity. Less information is available with regard to renal levels of cytokines in models of the metabolic syndrome or type II diabetes. Xu et al. [42] found increased mRNA expression for 2 cytokines, MCP-1 and IL-6, in renal cortical tissue obtained from similarly aged obese Zucker rats. These



Area under the curve (a.u.)

Mean ± sem	2-way ANOVA *P*-values
Lean C: 615 ± 24	Body type: < 0.001
Lean CAN: 528 ± 19*	Treatment: 0.081
Obese C: 792 ± 50*	Interaction: 0.005
Obese CAN: 1118 ± 137*#	

(a)



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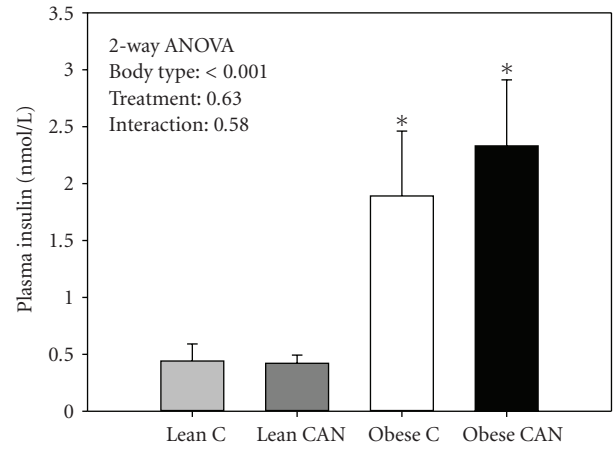

(c)

Figure 3: Metabolic function: (a) Glucose-tolerance test (GTT) performed in candesartan-treated (CAN) or untreated (c) lean and obese rats at 13 weeks of treatment ($n = 8$ per body type/treatment). Blood glucose levels measured at different time points in response to 50% dextrose solution (3 ml/kg · bw) given intraperitoneally. Area under the curve was higher for the obese rats compared to the lean, and a significant interaction was found between body type and treatment by 2-way ANOVA ($P < .05$). (b) Blood glucose and (c) plasma insulin levels at the end of 14 weeks of treatment ($n = 8$ per body type/treatment) were different between the body types by 2-way ANOVA ($P < .05$); 14 weeks of CAN treatment did not affect these levels. ∗ indicates a significant difference ($P < .05$) from lean control mean and # from obese control mean, by unpaired *t*-test.

levels were reduced by losartan (another ARB). In our study, we confirmed the increase at the protein level for MCP-1; however, we showed a decrease in IL-6 protein levels. In our rats, both of these cytokines were normalized by CAN. The difference between our study and that of Xu et al. [42] may have resulted from the difference in mRNA versus protein, or in the fact that we evaluated whole kidney, rather than only cortex. Another possibility is that the severity of the nephropathy may have affected the expression pattern. It is unclear whether their rats or ours had greater severity of renal disease at the time when samples were collected.

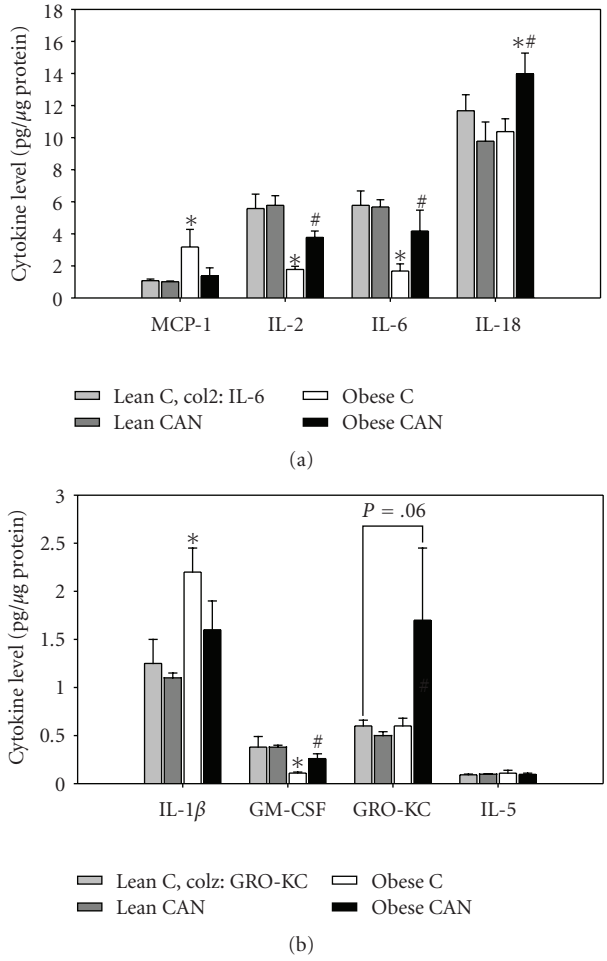

Figure 4: Mean kidney cytokine levels (all rats detectable) (a) higher level cytokines including: monocyte chemotactic protein-1 (MCP1), interleukin-2 (IL-2), interleukin-6 (IL-6), and interleukin-18 (IL-18); (b) lower level cytokines including: interleukin 1β(IL-1β), granulocyte macrophage colony-stimulating-factor (GM-CSF), growth regulated oncogene (GRO-KC), and interleukin-5 (IL-5) in whole kidney tissue homogenate from candesartan treated (CAN) or untreated (C) lean and obese rats at the end of 14 weeks of treatment ($n = 8$ per body type/treatment). $*$ indicates a significant difference ($P < .05$) from lean control mean and # from obese control mean, by unpaired t-test.

Overall, including IL-6, we found 9 cytokines that were significantly reduced in obese versus lean rat kidney. We suggest that renal protein levels of some cytokines or chemokines may actually decline with loss of epithelial cells and the progression of renal disease. In agreement, Waldherr et al. [43] reported that TNF-α, IFN-γ and IL-2 levels in the glomeruli were undetectable in the chronic form of human glomerulonephritis, while their levels were significantly increased in the acute form of the disease. Furthermore, a study examining the relationship between the expression of IL-6 mRNA and the degree of glomerular mesangial expansion in human diabetic nephropathy demonstrated that signal intensity for IL-6 mRNA was strongest in tissues from moderate mesangial expansion but was weak in those from mild and severe mesangial expansion [44]. These and our studies support the possibility that as renal disease progresses there is decompensation at the cellular level in the immune response, perhaps due to architectural or fibrotic changes. Further studies will be needed to address this possibility.

In further support of this hypothesis was the fact that a full 11 out of 14 cytokines in the obese rats were significantly different from lean rats in the untreated state and basically restored, or at least partially restored, by CAN. CAN attenuated renal damage, at least as gauged by reduced collagen and glycogen deposition and plasma creatinine. Furthermore, CAN had no significant effects on the lean rat cytokine profile, diminishing the possibility that other factors, for example, the fall in blood pressure with CAN as shown by us previously in both lean and obese rats [24], had any direct role on cytokine/chemokine levels.

Nevertheless, IL-18 and GRO-KC (which were not different between untreated lean and obese) were significantly increased in the treated obese rats only. We believe that hyperglycemia and/or slower plasma glucose clearance could be responsible for increased expression of these cytokines. High glucose levels have been demonstrated to increase the secretion of both IL-18 and GRO-KC [45]. Moreover, we showed that CAN treatment increased renal expression of transforming growth factor β1 (TGF-β1), but again, only in the obese rats. A potentially causative relationship between these two variables was recently demonstrated by Bani-Hani et al. [46] in IL-18-overexpressing mice; that is, TGF-β1 expression was reduced when IL-18 was neutralized by antibodies. IL-18 may have a facilitative role in glucose utilization by cells. Using IL-18 knockout mice, it has been demonstrated that lack of endogenous IL-18 results in obesity, insulin resistance, and hyperglycemia [47]. Furthermore, increased serum levels of IL-18 have been associated with insulin resistance and obesity in humans [48–50].

It is nevertheless somewhat surprising that TGF-β1 was increased quite dramatically in the obese CAN-treated rats, relative to all other groups, while our other evidence points to reduced epithelial-to-mesenchymal transition (EMT) and the development of fibrotic renal disease in obese rats with CAN treatment. It is possible that CAN simply delays renal disease in the obese Zucker rat and that increased TGF-1β may be transitory and occurred at an earlier time-point in the obese control rats. It is also possible that CAN is protective at a step down-stream of TGF-β1 with regard to matrix accumulation. Additional studies would need to be done to clarify this matter.

If we accept the possibility that CAN may have increased GRO-KC, IL-18, and TGF-β1 as a result of relative hyperglycemia in these rats, what was the mechanism for the impaired GTT? In fact, some evidence to contrary exists with regard to predicted systemic effects of AT1R blockade. ARBs, often prescribed to hypertensive subjects with the metabolic syndrome, have been demonstrated in several clinical studies to reduce the number of new onset cases of type II diabetes [51], improve glucose tolerance, and may prevent progressive beta-cell failure in diabetes [52, 53]. Tikellis et al. [53] found improved pancreatic islet morphology in Zucker diabetic

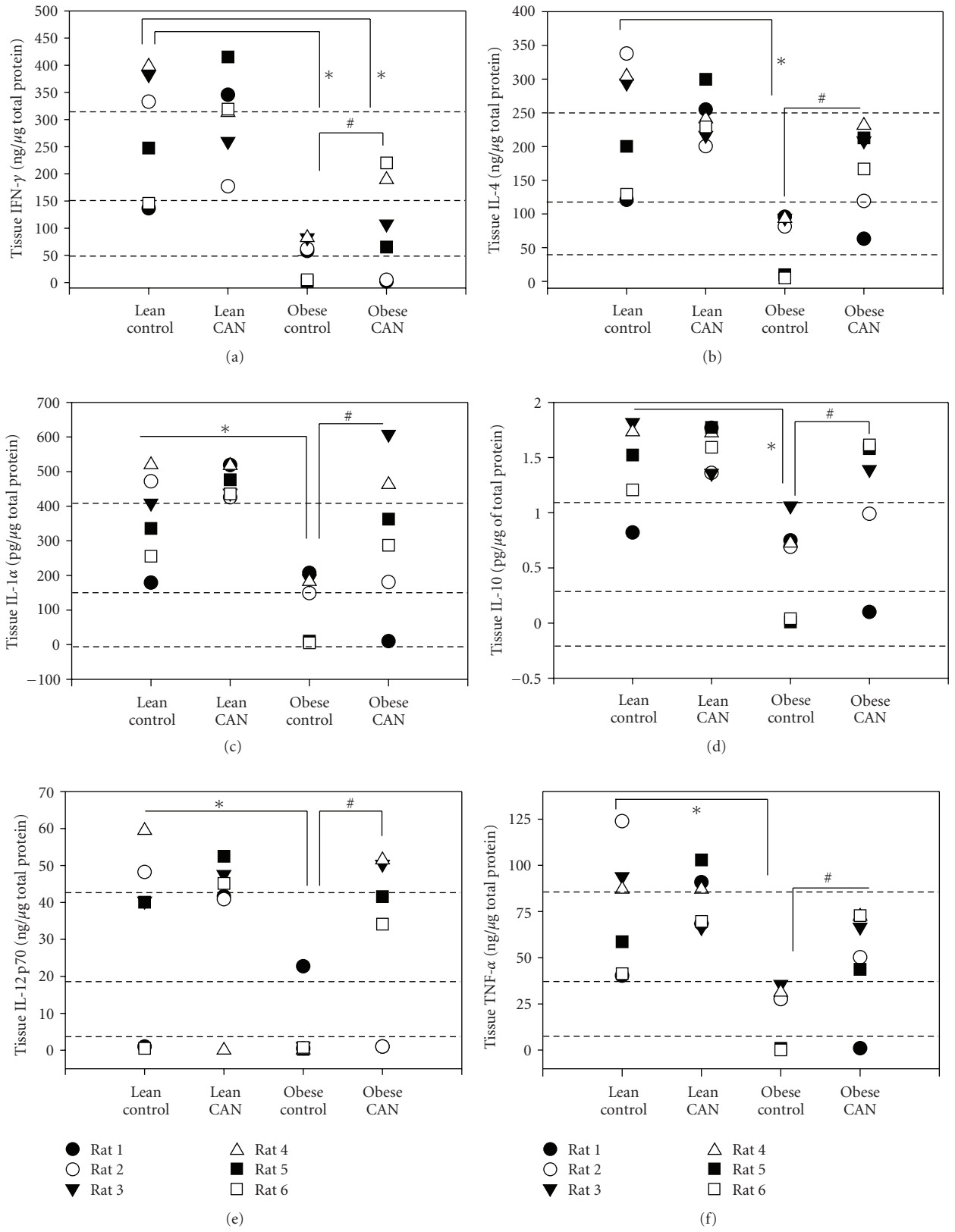

Figure 5: Individual kidney cytokine level (some rats undetectable) (a) interferon-γ (IFN-γ) (b) interleukin-4 (IL-4), (c) interleukin-1α (IL-1α), (d) interleukin 10 (IL-10), (e) interleukin 12p70, and (f) tumor necrosis factor α (TNF-α) in whole kidney tissue homogenate from candesartan-treated (CAN) or -untreated (c) lean and obese rats at the end of 14 weeks of treatment ($n = 8$ per body type/treatment). $*$ indicates a significant difference, $P < .05$, from lean control mean and # from obese control mean, by rank test on categorical assignments.

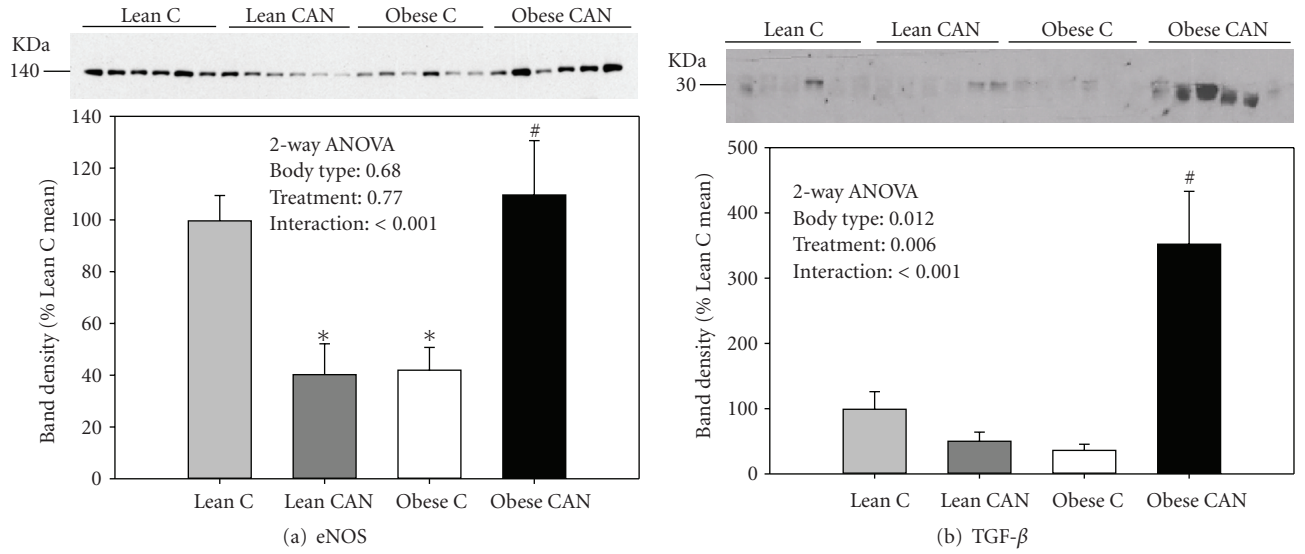

Figure 6: Western blotting of whole kidney eNOS and TGF-β. Representative immunoblot of whole kidney homogenates from -candesartan treated (CAN) or -untreated (C) lean and obese rats at the end of 14 weeks of treatment probed with (a) eNOS and (b) TGF-β antibodies, respectively. Equal amounts of total protein were loaded in each lane and each lane is loaded with a sample from a different rat. Below each blots is its densitometry summary ($n = 6$ rats/group). Results were analyzed by 2-way ANOVA and also by t-test. $*$ indicates a significant difference ($P < .05$) from lean control mean and # from obese control mean, by unpaired t-test.

fatty rats (ZDF), a substrain of the Zucker rat, that develops type II diabetes extremely early, after treatment with irbesartan (an ARB) or perindopril (an angiotensin converting enzyme inhibitor). Furthermore, candesartan was shown to improve GTT in high-fat fed Wistar rats [54]. These rats had increased expression of the peroxisomal proliferator activated receptor, subtype γ (PPAR-γ) in liver and adipose tissue, which they proposed may have been the mechanism for candesartan's effects [54].

Nonetheless, there are some clinical trials of 5 months and longer, in diabetic patients, which in agreement with our findings showed no improvement in metabolic parameters, including glucose tolerance with chronic ARB therapy [55–58]. In our study, the differences between treated- and untreated-obese rats were not large; in fact final blood glucose and insulin levels trended higher with CAN treatment, but were not statistically different and highly variable. What is clear is that they were not improved. However, surprisingly, GTT was significantly improved in the lean rats. Therefore, only obese rats responded in this somewhat negative fashion to chronic CAN therapy with regard to GTT. Glucose dose was administered intraperitoneally (ip) according to weight of the rats, but there were no significant differences in body weight between CAN-treated and control obese rats at the time of the GTT. It is possible that the lower BP in the CAN-treated rats somehow resulted in delayed uptake from the ip cavity, with subsequent delay in clearance. The blood glucose levels in the CAN-treated obese rats did not peak until 1.5 hours, while they were at their peak in the control obese rat at 30 minutes (Figure 3). However, the fall in BP in the lean rats did not affect time-of-peak for glucose, which occurred at the same time (15 minutes) for lean control and lean CAN-treated rats. It is possible that chronic CAN treatment negatively impacted the pancreatic release of insulin in the obese rats, for example, as a result of the low BP. Insulin levels were not measured during the GTT challenge; however, in the basal state they were higher (not lower) than control obese rats, suggesting relatively greater insulin resistance in these rats with the ability to compensate with hyperinsulinemia still intact. Thus, it appeared that they may have been more insulin resistant at the level of peripheral tissues.

Candesartan-treated obese rats also exhibited relative polyuria and polydipsia, despite improvement in many histological features of the kidney, and in general renal function. Increased urine volumes could be due to glucose-induced osmotic diuresis in the CAN-treated obese rats, further supporting impaired glucose handling and insulin resistance in these rats. In contrast, lean rats treated with CAN showed the opposite; that is, they had significantly reduced urine volumes with CAN. The mechanism(s) underlying reduced urine volume with CAN treatment in these lean rats is also unknown. Ang II has been shown to stimulate thirst via AT1R in the brain. Candesartan administered peripherally has been shown to block this effect [59]. Thus, it is possible that this is the mechanism underlying reduced water intake in the lean rats. We might speculate that this effect was masked in the obese due to thirst generated as a result of the osmotic diuresis.

Overall, CAN therapy was protective of the kidney both functionally and histologically. CAN was able to restore or normalize (to lean levels) aberrant renal levels of 11 of 14 cytokines measured. This may be critical in the continuation of adequate immune function in the kidney of the obese rat. In contrast to obese rats, CAN had no effects on renal cytokine levels in lean rats. Moreover, these protective

changes occurred despite candesartan's propensity to worsen glucose tolerance in the obese rats. Impaired GTT and increased levels of renal IL-18, GRO-KC, and TGF-β1 in the CAN-treated obese rats were puzzling, but of clear concern. The mechanisms underlying these effects require additional study.

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Review Article

Functional Food Targeting the Regulation of Obesity-Induced Inflammatory Responses and Pathologies

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Obesity is associated with a low-grade systemic chronic inflammatory state, characterized by the abnormal production of pro- and anti-inflammatory adipocytokines. It has been found that immune cells such as macrophages can infiltrate adipose tissue and are responsible for the majority of inflammatory cytokine production. Obesity-induced inflammation is considered a potential mechanism linking obesity to its related pathologies, such as insulin resistance, cardiovascular diseases, type-2 diabetes, and some immune disorders. Therefore, targeting obesity-related inflammatory components may be a useful strategy to prevent or ameliorate the development of such obesity-related diseases. It has been shown that several food components can modulate inflammatory responses in adipose tissue via various mechanisms, some of which are dependent on peroxisome proliferator-activated receptor γ (PPARγ), whereas others are independent on PPARγ, by attenuating signals of nuclear factor-κB (NF-κB) and/or c-Jun amino-terminal kinase (JNK). In this review, we introduce the beneficial effects of anti-inflammatory phytochemicals that can help prevent obesity-induced inflammatory responses and pathologies.

1. Introduction

Recently, more and more lines of evidence have accumulated that obesity is associated with low-grade chronic inflammation that is causally involved in the development of insulin resistance. Systemic inflammation is markedly evident in a number of human and mouse models of obesity, as determined by increased plasma levels of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1). These inflammatory cytokines are derived from obese adipose tissue [1], and recently, it has been found that not only adipocytes, but also immune cells, such as macrophages [2, 3] reside in adipose tissue, and that these cells may induce insulin resistance by promoting inflammation in these tissues. The major cause of the development of obesity and the consequent inflammatory disorders is the excess dietary fat intake or an imbalance between the intake and expenditure of energy. Overweight and obese patients may develop paradoxical nutritional deficiency from eating high energy foods with poor nutrient content; however, diet with a higher nutrient density reduces their weight and improves obesity-related inflammatory disorders [4]. This indicates that obesity-related pathologies can be prevented or improved by the intake of food containing components that can control inflammation in obese adipose tissues infiltrated with macrophages. In activated macrophages, inflammatory responses are regulated by master regulators of inflammation such as nuclear factor-κB (NF-κB) and c-Jun amino-terminal kinase (JNK) [5, 6]. Moreover, peroxisome proliferator-activated receptor-γ (PPARγ) is reported to attenuate inflammation in activated macrophages by interfering with NF-κB signaling [7]. Therefore, targeting these inflammatory regulators using food components may be a useful strategy to prevent or ameliorate the development of obesity-related diseases. Our group and other research groups have shown that several food components can modulate inflammatory responses in adipose tissue via various mechanisms, some of

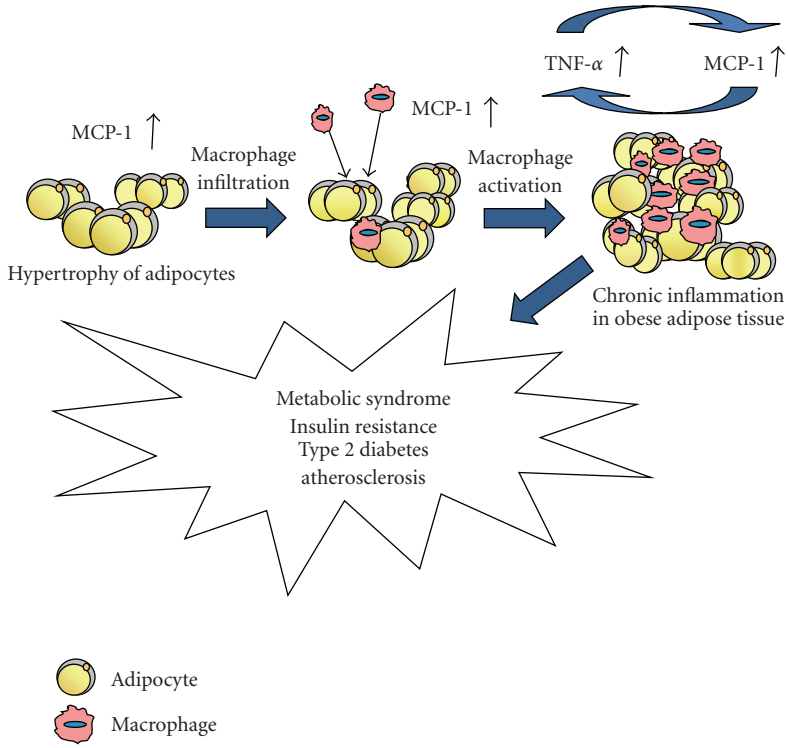

Figure 1: The development of vicious cycle of inflammation between adipocytes and macrophages in obese adipose tissue.

which are dependent on PPARγ, whereas others are PPARγ-independent, by attenuating NF-κB or JNK signaling. In this review, we introduce the beneficial effects of anti-inflammatory food components against obesity-induced inflammatory responses and pathologies.

2. Inflammatory Components Associated with Obesity and Related Pathologies

Adipose tissue is composed of adipocytes and stromal vascular cells containing various cell types such as preadipocytes, endothelial cells, fibroblasts, and numerous immune cells. In particular, macrophage infiltration into adipose tissue is prominent in obesity, and the number of macrophages in adipose tissue correlates with body mass index, adipose size, and the total amount of body fat [2, 3]. It has been suggested that adipose tissue-derived MCP-1, a CC chemokine that exhibits chemotactic properties on inflammatory cells, is the key factor for inducing macrophage infiltration into adipose tissue. The level of MCP-1 released by adipocytes is significantly greater in obese mice than in nonobese mice [8–10] and is markedly increased when adipocytes are cocultured with macrophages [11, 12]. MCP-1 from hypertrophic adipocytes in obese adipose tissue can also trigger macrophage infiltration into adipose tissue and subsequently activates macrophages to release inflammatory mediators such as TNF-α [10], which interferes with insulin signaling and induces fatty acid lipolysis in adipocytes. The concentrations of these fatty acids, particularly saturated free fatty acids, are reported to be elevated in obesity [13] and directly induce inflammatory responses in macrophages via toll-like receptor 4 (TLR4), the lipopolysaccharide receptor [14, 15]. The NF-κB and JNK pathways represent important modulators of inflammatory gene expression downstream of TLR4 in many cell types, including macrophages [11, 16, 17]. In this way, adipocytes and macrophages interact in a paracrine manner and create a vicious cycle of inflammation that augments the inflammatory changes and insulin resistance in obese adipose tissue [11] (Figure 1).

3. Strategy to Prevent Inflammatory Responses and Insulin Resistance in Obese Adipose Tissues by Food Components

Inflammatory responses in obese adipose tissues are regulated by many transcriptional factors. NF-κB and JNK represent important modulators of inflammatory gene expression downstream of TLR4 in adipose tissues, suggesting that food components interfering with the TLR4/NF-κB or TLR4/JNK axis could be useful to prevent the onset of insulin resistance in obese patients (Figure 2).

Furthermore, PPARγ, a member of the nuclear receptor superfamily activated by ligands, also plays an important role in inflammation [18, 19]. Thiazolidinediones (TZDs), synthetic ligands for PPARγ, suppress the production of proinflammatory cytokines including TNF-α in LPS-stimulated macrophages [7]. In addition to the anti-inflammatory effect, TZDs regulate the mRNA expression of genes involved in lipid metabolism in macrophages and suppress their transformation into foam cells [7, 20]. On the other hand,

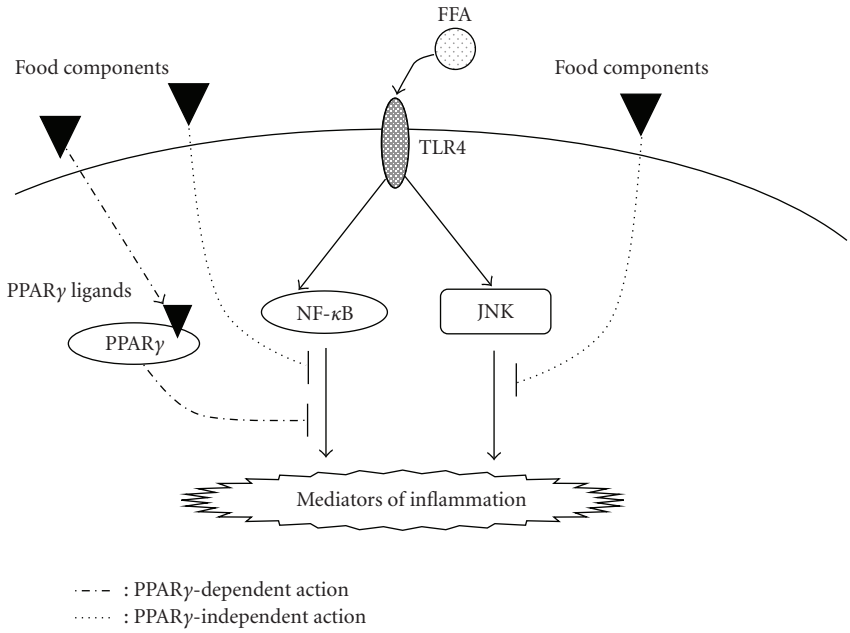

Figure 2: Signaling pathway of inflammatory gene expressions in obese adipose tissue and the strategy to prevent the obese-related pathologies by food components.

TZDs have been widely used as antidiabetic drugs, which activate PPARγ to resulting in the promotion of adipocyte differentiation [21]. TZDs not only stimulate glucose uptake into differentiated adipocytes but also induce the production of adiponectin, an insulin-sensitivity-promoting factor [22], and suppression of TNF-α through the PPARγ activation in adipocytes [23]. Thus, food components that act as ligands for PPARγ can show multiple effects, including anti-diabetes and anti-inflammatory effects. Currently, two different molecular mechanisms have been proposed by which the anti-inflammatory actions of PPARγ are in effect: (1) via interference with proinflammatory transcription factors including NF-κB [7], and (2) by preventing the removal of corepressor complexes from gene promoter regions resulting in the suppression of inflammatory gene transcription [24].

4. Food Components That Regulate Inflammation in Obese Adipose Tissue

On the basis of the strategy suggested above, our research group focused on the PPARγ-dependent or PPARγ-independent mechanisms to suppress the inflammatory mediators secreted by obese adipose tissues. For the screening of food components related to the former mechanism, our research group used the sensitive PPARγ ligand assay system developed by modifying the luciferase reporter assay system [25] and has found several phytochemicals that act as PPARγ agonists (Table 1). To evaluate the characteristics of food components that prevent obesity-induced inflammatory responses, we used the coculture system of adipocytes and macrophages, which is an *in vitro* model of obese adipose tissue infiltrated by macrophages (Figure 3).

Table 1: Phytochemicals that regulate obesity-induced inflammation.

Compound	Origin	PPARγ dependency
Abietic acid	Pine rosin	dependent
Anthocyanin	Red/Purplish Fruit	independent
Auraptene	Citrus Fruit	dependent
Capsaicin	Hot pepper	dependent
Dehydroabietic acid	Pine rosin	dependent
Diosgenin	Fenugreek, Yam	independent
6-Gingerol	Ginger	independent
Isohumulone	Humulus lupulus hop	dependent
Isoprenoid	Herb	dependent
Luteolin	Herb, Spice	independent
Naringenin	Citrus Fruit	independent
Naringenin chalcone	Tomato peel	independent
PUFA	Fish oil	independent
Resveratrol	Red wine	dependent
6-Shogaol	Ginger	dependent

4.1. PPARγ-Dependent Action. Spices are derived from plants cultivated in temperate and tropical zones, and many of them have antioxidant, anticancer, antiobesity, and anti-inflammatory activities [26–28]. Several anti-inflammatory spice-derived components are reported to modulate inflammatory responses in adipose tissue and therefore improve obesity-related pathologies such as insulin resistance [29, 30].

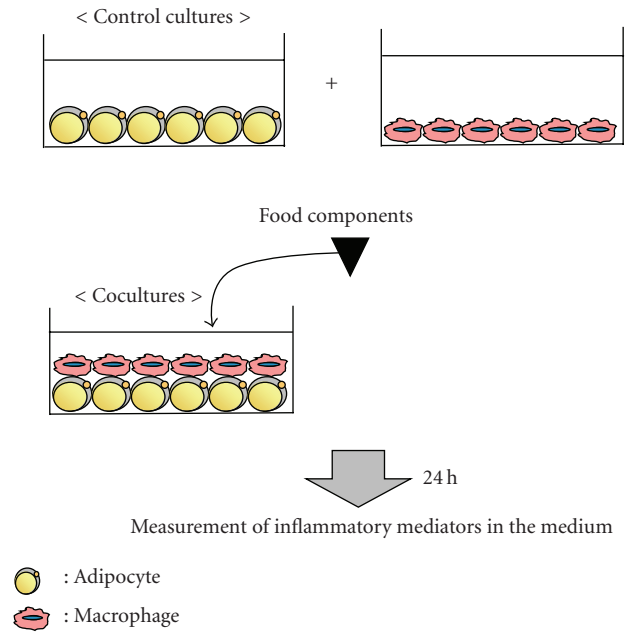

Figure 3: Coculture system of adipocytes and macrophages for the screening for anti-inflammatory food components.

Capsaicin, a spicy ingredient of hot peppers, has not only metabolic properties to induce thermogenesis and fat oxidation [26, 28] but also anti-inflammatory properties [31]. In the adipose tissue or adipocyte culture system, capsaicin inhibits the expression and secretion of IL-6 and MCP-1 from the adipose tissues and adipocytes of obese mice, whereas it enhances the expressions of the adiponectin gene and protein [29]. These actions of capsaicin are associated with NF-κB inactivation, which is probably mediated by PPARγ activation [29]. Moreover, capsaicin suppresses not only macrophage migration induced in an adipose-tissue-conditioned medium but also its activation to release proinflammatory mediators. It is also demonstrated that capsaicin administration *in vivo* improves obesity-induced insulin resistance [29].

Ginger, which is the rhizome of the plant *Zingiber officinale* Roscoe, is widely used as a spice and herbal medicine. 6-Shogaol is the main ginger-derived component, which has potent anti-inflammatory activities [32, 33]. Because 6-shogaol is a potent agonist of PPARγ, it not only enhances the expressions of adiponectin and aP2 but also inhibits the TNF-α-induced downregulation of adiponectin expression in adipocytes [34].

Isoprenoids (terpenoids), which are present in many dietary and herbal plants [35], exhibit many biological effects: antitumor proliferation, anti-hypercholesteremia, and anti-diabetes [35–37]. Abietic acid (AA) and one of its derivatives, dehydroabietic acid (DAA), are diterpenes, which are both the major components of the rosin fraction of oleoresin synthesized by conifer species, such as grand fir (*Abies grandis*) and lodgepole pine (*Pinus contorta*) [38]. We have found that both AA and DAA have anti-inflammatory effects on macrophages, which are mediated by PPARγ activation [25]. When DAA was administered with a high-fat diet to obese diabetic KK-Ay mice, DAA suppressed the production of proinflammatory mediators such as MCP-1 and TNF-α, increased that of adiponectin, and reduced the infiltration of macrophages into the adipose tissues of HFD-fed mice [39]. DAA can also strongly activate PPARα, which is mainly involved in the control of lipid metabolism [40], and the fact that PPARα agonists such as Wy-14643 can suppress inflammation in adipose tissues [41] suggests that DAA as a PPARα/γ dual agonist is a valuable medicinal food-derived component for improving the inflammation caused by obesity and for controlling metabolic syndrome.

Auraptene (a monoterpene derivative), a citrus fruit compound contained mainly in the peel, is also a PPARα/γ dual agonist [42, 43]. In adipocytes, auraptene regulates the transcription of PPARγ target genes, induces the expression and secretion of adiponectin, and inhibits those of MCP-1 [42]. It is also observed that auraptene can suppress the inflammatory changes between adipocytes and macrophages and the macrophage infiltration into obese adipose tissues (Lin et al. unpublished data). Several reports have indicated that coapplication of PPARα and PPARγ agonists or treatment with dual agonists causes more efficient glucose uptake into adipocytes to decrease the blood glucose level without the increase in body weight [41, 44]. Further *in vivo* investigations are necessary to elucidate the inhibitory effect of auraptene on chronic systemic inflammation induced by obesity.

4.2. PPARγ-Independent Action. Flavonoid is a general term for plant metabolites that have a C6-C3-C6 structure. Chalcone is the first product in the flavonoid biosynthesis

pathway, which is catalyzed by chalcone isomerase, resulting in the flavanone naringenin. Most flavonoids are then metabolized to flavone, dihydroflavonol, flavonol, leucoanthocyanidin, catechin, and anthocyanidin by oxidation-reduction reaction. Over 4,000 flavonoids have been identified, many of which occur in vegetables and fruits. These flavonoids have been reported to have antiviral, antiallergic, antiplatelet, anti-inflammatory, antitumor, and antioxidant activities, and recently, they have attracted considerable interest because of their potential beneficial effects on obesity and metabolic syndromes.

Luteolin, a flavone that is present in medicinal plants and in some vegetables and spices, has been reported to exhibit antioxidant, anti-inflammatory, and antiallergy functions [45]. Recently, we have found that luteolin also inhibits low-grade chronic inflammation induced during the coculture of adipocytes and macrophages [17]. Luteolin does not affect I-κB-α degradation and thus may not affect the NF-κB activation. However, it inhibits the phosphorylation of JNK in the macrophages activated by the conditioned medium derived from adipocytes [17]. Because luteolin is not a PPARγ agonist (Ando et al. unpublished data), luteolin may act on JNK directly or indirectly via a PPARγ-independent mechanism.

Using the coculture system of adipocytes and macrophages, we have also found similar effects of naringenin chalcone, a type of flavonoid accumulated in tomato peels. Naringenin chalcone has only been reported as having antiallergic activities [46]; therefore, we examined its effect on the inflammatory changes associated with the interaction of adipocytes and macrophages. As in the case of luteolin, naringenin chalcone also suppresses the production of inflammatory mediators induced by the coculture of adipocytes and macrophages [12]. The flavanone naringenin, which is abundant in citrus fruits, also inhibits coculture-induced inflammation; however, the suppressive effect is more notable in naringenin chalcone [12]. However, unlike luteolin, naringenin chalcone and naringenin partly inhibit the degradation of I-κB-α [12] and suppress the macrophage infiltration to hypertrophied adipocytes (Hirai et al. unpublished data). These three flavonoids do not serve as agonists of PPARγ in the luciferase reporter assay (Hirai et al. unpublished data); thus, it is considered that they also affect the signaling molecules downstream of TLR4 directly or indirectly but independently of PPARγ activation in macrophages.

Anthocyanins, another type of flavonoid found in red/purplish fruits and vegetables, including purple grapes, apples, blueberries, egg apples, and beans, are well-known antioxidants. These flavonoids have also been shown to have anti-inflammatory activity in obese adipose tissues, which is mediated by PPARγ-independent mechanisms [47, 48]. Moreover, cyanidin 3-glucoside (C3G), a typical anthocyanin, downregulates the retinol binding protein 4, which is known to ameliorate insulin sensitivity in the white adipose tissue of diabetic KK-Ay mice [49]. Therefore, the C3G-induced improvement of insulin sensitivity may be associated with the inhibition of inflammatory mediators and stimulation of AMPK activity via PPARγ-independent mechanisms [48].

Aside from flavonoids, a saponin aglycon, diosgenin, is also found to suppress the inflammatory mediators induced by the interaction of adipocytes and macrophages. Diosgenin is found in a variety of plants including fenugreek (*Trigonella foenum-graecum*) and roots of wild yam (*Dioscorea villosa*), and its extracts have been traditionally used to treat diabetes [50] and hypercholesterolemia [51]. Many researchers have shown that diosgenin has various biological functions, including anti-inflammation [52]. In our coculture system, diosgenin also inhibited the inflammatory changes via the downregulation of I-κB-α degradation and JNK activation [53], which is independent of PPARγ activation (Uemura et al. unpublished data).

6-Gingerol is another main ginger-derived component besides 6-shogaol. The structures of these two components are very similar and both are reported to inhibit TNF-α-mediated suppression of adiponectin in adipocytes; however, the mechanisms of their inhibitory effects are different; 6-gingerol inhibits JNK signaling pathways in TNF-α-induced adipocytes without affecting PPARγ transactivation, whereas the anti-inflammatory action of 6-shogaol is PPARγ-dependent [54]. These results suggest that slight structural differences may affect the affinity for PPARγ and the inhibition of the JNK signaling pathways.

Although saturated fatty acids directly induce inflammatory responses in macrophages, long-chain ω-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are known as antiobesity and anti-inflammatory factors. Fish oil containing high concentrations of DHA and EPA is considered a good source of ω-3 PUFA. The prevention of high-fat or high-energy-diet-induced adipose tissue inflammation and remodeling by long-chain ω-3 PUFA is reported to be involved in PPARγ activation [55, 56]. However, the anti-inflammatory mechanisms of PUFA action are diverse and involve PPARγ-independent effects [57]. Furthermore, PUFA needs many cofactors such as folic acid, vitamins, tetrahydrobiopterin, minerals, and L-arginine for their physiological actions [58]. Hence, these cofactors should also be provided in adequate amounts to bring about the anti-inflammatory actions of ω-3 PUFA in obese adipose tissues.

5. Conclusions/Outlook

A growing number of studies strongly support that obesity-induced inflammation plays an important role in the development of obesity-related pathologies such as insulin resistance, cardiovascular diseases, type-2 diabetes, and some immune disorders. NF-κB and JNK are important modulators of inflammatory gene expression downstream of TLR4 in obese adipose tissues, which are regulated by PPARγ. All the food components described above are beneficial phytochemicals that ameliorate obesity-induced inflammatory responses and pathologies by suppressing the inflammatory signaling in a PPARγ-dependent or PPARγ-independent manner. In particular, PPARγ agonists can directly reduce adipocyte size and induce the expression of anti-inflammatory cytokines, such as adiponectin [23].

Moreover, PPARγ agonists have recently been reported to cause the polarization of adipose tissue macrophages to M2 phenotypes, resulting in the secretion of anti-inflammatory cytokines [59]. Thus, food components with PPARγ agonistic activities may also contribute to the improvement of obesity-induced inflammation via adipose tissue remodeling associated with the phenotype switch of macrophages. Recently, it has been reported that a combination of bioactive compounds is very effective *in vivo* [60]. In particular, a combination of compounds exhibiting different mechanisms by which anti-inflammatory effects are exerted seems to be most efficient. Therefore, all the described phytochemicals in this review, which act as PPARγ agonists, may be suitable for the treatment of metabolic syndrome together with other compounds that can suppress inflammatory responses in a PPARγ-independent manner, by directly inhibiting NF-κB or JNK signaling. As shown in Table 1, citrus fruits including oranges, grapefruits, lemons, and some limes, and fish oil from blue-skin fish such as sardine, herring, and albacore tuna, are the most available anti-inflammatory foods in the market. On the other hand, our daily intake of spices and herbs are still limited. Further studies on the effective amounts and forms of intake will help promote the development of all these functional foods in the world.

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Research Article

Human Lipoxygenase Pathway Gene Variation and Association with Markers of Subclinical Atherosclerosis in the Diabetes Heart Study

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Aims. Genes of the 5-lipoxygenase pathway are compelling candidates for atherosclerosis. We hypothesize that polymorphisms in *ALOX12, ALOX15, ALOX5*, and *ALOX5AP* genes are associated with subclinical atherosclerosis in multiple vascular beds. *Methods*. Families with two or more siblings with type 2 diabetes and their nondiabetic siblings were studied as part of the Diabetes Heart Study (DHS). European American diabetic ($n = 828$) and nondiabetic ($n = 170$) siblings were genotyped for SNPs in the *ALOX12, ALOX15, ALOX5*, and *ALOX5AP* genes. Subclinical measures of atherosclerosis (IMT, coronary (CorCP), carotid (CarCP) and aortic (AorCP) calcified plaque) were obtained. *Results*. Associations were observed between *ALOX12* with CorCP, *ALOX5* with CorCP, AorCP, and IMT, and *ALOX5AP* with CorCP and CarCP, independent of known epidemiologic risk factors. Further, lipoxygenase pathway SNPs that were associated with measures of atherosclerosis were associated with markers of inflammation (CRP, ICAM-1) and calcification (MGP). *Conclusions*. Polymorphisms within *ALOX12, ALOX5*, and *ALOX5AP* are genetically associated with subclinical atherosclerosis and with biomarkers of disease in families with type 2 diabetes. These results suggest that variants in lipoxygenase pathway genes may have pleiotropic effects on multiple components that determine risk of cardiovascular disease.

1. Introduction

Atherosclerosis is thought to be the result of chronic inflammation of the artery wall although the pathways and factors that initiate and modulate the inflammatory response in atherosclerosis have yet to be completely resolved [1]. Metabolites of arachidonic acid are strong candidates that are recognized for their inflammatory properties. The mouse 5-LO gene, *ALOX5*, has been shown to contribute to the development of atherosclerosis [2]. Variants in the human homologue (*ALOX5*) are associated with carotid artery intima-media thickness (IMT) [3]. FLAP (5-lipoxygenase activating protein), encoded by the *ALOX5AP* gene, likely acts as an arachidonic acid-binding

and transfer protein to facilitate 5LO activity [4]. Single SNPs and haplotypes of *ALOX5AP* have been associated with myocardial infarction in multiple populations [5–7].

Human 12-lipoxygenase (encoded by *ALOX12*) and 15-lipoxygenase (encoded by *ALOX15*) have been localized to atherosclerotic plaques, suggesting that 12/15LO activity is involved in the development of atherosclerosis [8–10]. Overexpression of human 15-LO in mouse vascular endothelial cells increased markers of atherosclerosis [11]. Human aortic endothelial cells cultured in chronically high glucose levels results in elevated levels of 12S-HETE, suggesting activation of lipoxygenase pathways [12, 13].

The current research was motivated by the role of lipoxygenase pathway gene products in inflammation, initiation/progression of atherosclerosis, and their modulation of expression by glucose. The *ALOX12*, *ALOX15*, *ALOX5,* and *ALOX5AP* genes represent strong candidates for atherosclerosis risk, especially in the context of type 2 diabetes. We have assessed genetic variants (SNPs) in lipoxygenase pathway genes for evidence of association with markers of subclinical atherosclerosis (intima-media wall thickness [IMT], carotid artery calcified plaque [CarCP], coronary artery calcified plaque [CorCP], and aortic calcified plaque [AorCP]) and biomarkers (e.g., ICAM-1, E-selectin, CRP) in participants of the Diabetes Heart Study (DHS).

2. Patients and Methods

2.1. Participants. Recruitment and phenotyping of Diabetes Heart Study (DHS) participants have been previously described [14–16]. Siblings concordant for type 2 diabetes were recruited if they had no evidence of renal insufficiency, as were all available nondiabetic siblings. Participant examinations were conducted in the General Clinical Research Center of the Wake Forest University School of Medicine, and included interviews for medical history, medication use and health behaviors, anthropometry, resting blood pressure, a fasting blood sampling, and a spot urine collection. Only Caucasian participants were included in this report, due to small sample size and limited statistical power in the African-American cohort. All study protocols were approved by the Institutional Review Board of Wake Forest University School of Medicine. All participants provided written informed consent.

2.2. Measurements. Intima-media thickness (IMT) of the common carotid artery was measured by high-resolution B-mode ultrasonography with a 7.5-MHz transducer and a Biosound Esaote (AU5) ultrasound machine [14]. The mean of up to 20 common carotid IMT estimates was used as the phenotypic. Calcified plaque was measured in the carotid and coronary arteries and the aorta using single and multidetector CT systems that employ a standardized protocol which includes CT scanner phantom testing based on those currently implemented in the National Heart Lung and Blood Institute's (NHLBI) Multiethnic Study of Atherosclerosis (MESA) studies [15, 17].

2.3. Molecular Genetics. Total genomic DNA was purified from whole blood samples obtained from subjects using PUREGENE DNA isolation kit (Gentra, Inc., Minneapolis, MN). DNA was quantitated using standardized fluorometric readings on a Hoefer DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc., San Francisco, CA). Each sample was diluted to a final concentration of 5 ng/μL. Single nucleotide polymorphisms (SNPs) were identified for *ALOX12* (17p13.1), *ALOX15* (17p13.3), *ALOX5* (10q11.2), and *ALOX5AP* (13q12) and were chosen for genotyping in order to provide coverage of linkage disequilibrium (LD) blocks using the Program in Genome Applications (PGA, University of Washington) with $r^2 < 0.8$ and minor allele frequency (MAF) greater than 5%. As much of the HapMap data were not available at the time of the study, relatively few SNPs were available for each locus that passed the selection criteria and were suitable for the genotyping platform. All SNP genotypes were determined using a MassARRAY SNP genotyping system (Sequenom, Inc., San Diego, CA).

2.4. Statistical Analyses. The sample means, standard deviations (SD), and medians were computed on continuous variables, while proportions were determined for discrete variables in those DHS participants who contributed to the genetic analyses. Variables were transformed, when required, to meet normality assumptions. A series of generalized estimating equations (GEE) [18] assuming exchangeable correlation structure and using the empirical estimate of the variance (to adjust for familial correlation induced by selection of related individuals in sibships) was used for assessment of the effects of diabetes status on clinical variables.

2.5. Statistical Genetic Analyses. Allele and genotype frequencies were determined with unrelated subjects (a single member from each family) and tested for departure from Hardy-Weinberg proportions using a chi-square test (deviations with $P < .001$ were considered significant). Association between each individual SNP and each phenotype was determined using the quantitative pedigree disequilibrium test (QPDT) [19]. The QPDT method reduces the effect of undetected stratification bias as transmission, rather than frequency of specific SNP alleles, is used in estimating the association between SNP and phenotype. All association analyses were conducted with adjustment for known epidemiologic risk factors of atherosclerosis (age, gender, diabetes status, smoking, BMI, use of aspirin, estrogen (women only), and lipid-lowering and hypertension medications).

For any SNP that exhibited a significant association with an atherosclerosis phenotype, additional SNPs within the LD block were examined (when available). QPDT SNP haplotypes were determined using the EM algorithm. QPDT analyses were applied to these haplotypes, adjusting for the same risk factors as in the single SNP analyses. Genotype-specific means, medians, and number of subjects within each genotypic class and for each measure of atherosclerosis were computed under a specific genetic model. Analyses of

biomarkers (e.g., ICAM-1, E-selectin, CRP) were performed using the same analytic strategy.

3. Results

3.1. Patient Characteristics. DNA was collected from 998 European-American subjects from 368 families. Demographic characteristics of participants are shown in Table 1. The age at ascertainment of diabetic cases (62.0 ± 9.3 years) was slightly older than the nondiabetic siblings (59.5 ± 10.0 years). Duration of diabetes was 10.4 ± 7.1 years, reflecting a relatively early age at onset of type 2 diabetes (~52 years). BMI was higher in diabetic siblings (32.4 ± 6.7 kg/m^2) than in nondiabetic siblings (28.9 ± 5.2 kg/m^2). The nondiabetic subjects were, on average, overweight (as defined by a BMI between 25–29.9 kg/m^2) and approaching obese. There were few differences in lipid (total cholesterol, HDL, LDL) profiles between diabetic and nondiabetic subjects, although lipid lowering agents were used more commonly in the diabetic subjects (45.4%) than in the nondiabetic subjects (27.8%). Current and past smoking was highly prevalent in both diabetic (59.4%) and nondiabetic (57.4%) participants.

Cardiovascular characteristics of the DHS participants are shown in Table 2. Prevalence of CVD and extent of atherosclerosis (CorCP, CarCP, AorCP, IMT) varied by location (vascular bed) and diabetes. Diabetic participants had greater prevalence of all events/procedures (heart attack, angina, stroke, CABG, angioplasty) than nondiabetic siblings. Diabetic participants had significantly more calcified plaque in the coronary (1425 ± 29 versus 551 ± 13, mean ± stderr), carotid (374 ± 25 versus 168 ± 35), and aorta (3995 ± 166 versus 2342 ± 309) than nondiabetic siblings; however, there was no significant difference in IMT with respect to diabetes status (0.68 ± 0.01 in diabetics, 0.64 ± 0.01 in nondiabetics).

3.2. Lipoxygenase Pathway Gene SNPs. Allele and genotype frequencies of SNPs in *ALOX12*, *ALOX15*, *ALOX5,* and *ALOX5AP* were assessed for deviation from Hardy-Weinberg expectations in unrelated probands (see Supplementary Table 1 in Supplementary Material available online at doi:10.1155/2010/170153). No SNP exhibited significant ($P < .001$) deviation from Hardy-Weinberg equilibrium. All SNPs were maintained in subsequent analyses. The distribution of SNPs provided coverage within the block structure of the genes, as well as providing additional coverage (either 3′ or 5′) of the regions adjacent to the lipoxygenase pathway genes.

3.3. Lipoxygenase Pathway Genes and Coronary Calcified Plaque (CorCP). None of the 17 tagging SNPs in the lipoxygenase pathway genes were significantly ($P < .05$) associated with CorCP (Table 3).

In exploratory analyses, four SNPs exhibited suggestive association ($P < .10$) with CorCP, one in *ALOX12* (rs2271316, $P = .061$, 3′ of the gene), one in *ALOX5* (rs2115819, $P = .090$, intron 3), and two in *ALOX5AP* (rs9506352, $P = .097$, intron 2; rs4769060, $P = .073$, intron 4). In *ALOX12*, another SNP near rs2271316 was identified (rs1042357, exon 8) and was genotyped. This SNP (rs1042357) is only 10 kb from rs2271316. The association of rs1042357 with variation in CorCP was not significant ($P = .211$) and the two-SNP haplotype was also not significant ($P = .157$). Hence, the *ALOX12* marginal association was not confirmed. In *ALOX5*, two additional SNPs were identified (rs1369214, intron 3; rs11239524, intron 4) and were genotyped. These two SNPs bound the *ALOX5* marginally associated SNP (rs2115819, intron 3). The *ALOX5* SNP rs1369214 is only 360 bp from rs2115819, while the *ALOX5* SNP rs11239524 is ~11 kb from *ALOX5* SNP rs2115819. The association between CorCP with rs1369214 was similar in magnitude ($P = .098$), while the association between CorCP with rs11239524 was much stronger ($P = .027$). Two- and three-SNP haplotypes were not more strongly associated than either the original SNP (rs2115819 in intron 3) or the SNP in intron 4 (rs11239524), suggesting that there may be an effect of *ALOX5* on variation of CorCP in intron 4 worthy of further examination. In *ALOX5AP*, two SNPs were identified as having a marginal effect on variation in CorCP (rs9506352 in intron 2 and rs4769060 in intron 4). Since the distance between these two SNPs is ~17 kb, three additional SNPs (rs4769874 in intron 3, rs9315048 in intron 3, and rs12019512 in intron 4) were genotyped in these samples. The three SNPs were not significantly associated with CorCP, and analyses of two-SNP haplotypes failed to increase evidence of association. Thus, the exploratory genotyping and data analyses for *ALOX5AP* SNP did not increase evidence of association with CorCP.

Table 1: Clinical and laboratory characteristics of Caucasian DHS participants.

	Diabetic patients ($n = 828$)	Nondiabetic patients ($n = 170$)
Age (years)	62.0 ± 9.3	59.5 ± 10.0
Female (%)	51.20%	61.80%
Diabetes duration (years)	10.4 ± 7.1	
BMI (kg/m^2)	32.4 ± 6.7	28.9 ± 5.2
Cholesterol (mmol/L)	4.8 ± 1.1	5.0 ± 0.9
HDL (mmol/L)	1.1 ± 0.3	1.2 ± 0.4
LDL (mmol/L)	2.7 ± 0.8	2.9 ± 0.8
CRP (mg/L)	5.1 ± 8.3	4.1 ± 6.5
Smoking (current/past) (%)	59.40%	57.40%
Lipid lowering (%)	45.40%	27.80%
Estrogen (%, in women)	26.60%	34.80%
Aspirin (%)	57.60%	53.30%
Hypertension (%)	79.40%	46.50%

3.4. Lipoxygenase Pathway Genes and Carotid Calcified Plaque (CarCP). No SNPs in the lipoxygenase pathway genes were significantly ($P < .05$) associated with CarCP (Table 3).

In exploratory analyses, one SNP in *ALOX5AP* (rs10507391, $P = .085$, intron 1) exhibited suggestive

Table 2: Prevalence of CVD (%) and mean (± SD) for atherosclerosis phenotypes (CorCP, CarCP, AorCP, IMT) for Caucasian participants in the Diabetes Heart Study (DHS).

	Diabetic patients (n = 828)	Nondiabetic patients (n = 170)
Prevalent CVD		
Heart attack (%)	22.10%	8.30%
Angina (%)	20.40%	8.70%
Stroke (%)	10.40%	5.40%
CABG (%)	15.60%	7.70%
Angioplasty (%)	17.10%	5.30%
Endarterectomy (%)	2.30%	2.40%
Vascular Imaging		
Coronary calcified plaque	1425 ± 2637	551 ± 1187
Carotid calcified plaque	374 ± 726	168 ± 457
Aortic calcified plaque	3995 ± 4792	2342 ± 4033
Carotid IMT (mm)	0.68 ± 0.13	0.64 ± 0.12

association ($P < .10$). Two tagging SNPs adjacent to rs10507391 (rs4769055 and rs9551960) span a 7 kb region in intron 1 of *ALOX5AP*. Two-SNP haplotype analyses identified the rs10507391-rs9551960 haplotype as significantly associated with variation in CarCP ($P = .002$). The rs10507391-rs9551960-rs9506352 three-SNP haplotype was also strongly associated with CarCP ($P = .003$), while the rs4769055-rs10507391-rs9551960 haplotype was not associated ($P = 374$). These data suggest that the *ALOX5AP* effect on CarCP may reside between rs10507391 (intron 1) and rs9506352 (intron 2). No additional genotyping of other *ALOX5AP* SNPs was performed.

3.5. Lipoxygenase Pathway Genes and Aortic Calcified Plaque (AorCP). No tagging SNP in the four lipoxygenase pathway genes were significantly associated ($P < .05$) with AorCP (Table 3).

In exploratory analyses, one *ALOX5* SNP (rs2115819 in intron 3) exhibited the strongest ($P = .108$) association. The rs2115819 SNP was also associated with variation in CorCP. Two additional SNPs near the *ALOX5* rs2115819 SNP were identified for examination. Genotyping these two adjacent SNPs in *ALOX5* (rs1369214, intron 3; rs11239524, intron 4) failed to provide evidence of association, either in analyses of single SNPs or two- and three-SNP haplotypes. Thus, the marginal association with AorCP was not confirmed.

3.6. Lipoxygenase Pathway Genes and Intima-Media Thickness (IMT). None of the tagging SNPs in the lipoxygenase pathway genes were significantly ($P < .05$) associated with IMT (Table 3).

In exploratory analyses, one *ALOX5* SNP (rs3780906 in intron 6) was marginally associated ($P = .090$) with IMT. Two additional *ALOX5* SNPs (rs3780901 in intron 4 and rs1059696 in intron 7) were identified that were near the *ALOX5* rs3780901 SNP. These SNPs were genotyped and tested for association. Neither bordering SNP was significantly associated with IMT (rs3780901, $P = .871$; rs1059696, $P = .746$). The distance spanned in *ALOX5* by the three SNPs was 17 kb. The two-SNP haplotype formed by rs3780901-rs3780906 (12 kb between intron 4 and intron 6) was significantly associated with IMT ($P = .047$). Further, the three-SNP haplotype (rs3780901-rs3780906-rs1059696) was strongly associated with IMT ($P = .019$). Thus, this region in *ALOX5* may be worthy of further examination for association with IMT.

3.7. Phenotypic Effect of Lipoxygenase Pathway Gene Variants on Measures of Atherosclerosis. Genotype-specific means, medians, and number of subjects within each genotypic class and for each measure of subclinical atherosclerosis are presented in Table 4. Although no statistically significant effects were observed based upon comparisons of genotypic means (due to large variances of measured phenotypes and long-tailed phenotypic distributions), there were interesting trends in comparison of genotypic medians.

For the *ALOX5* rs2115819 SNP with CorCP and AorCP, the genotype-specific means suggested an inheritance pattern consistent with a dominant effect of allele "1", with the group mean (±SE) for the combined (1/1 and 2/1) genotypes having significantly less CorCP (1127 ± 95) and AorCP (3026 ± 183) than the 2/2 genotype (1540 ± 135 and 5318 ± 540, resp.). The "dominant" pattern is also seen for the *ALOX12* rs2271316 SNP [1/1 and 2/1 genotypes having significantly less CorCP (1097 ± 97) than the 2/2 genotype (1423 ± 216)] and for the *ALOX5AP* rs9506352 SNP [1/1 and 2/1 genotypes having significantly less CorCP (1145 ± 87) than the 2/2 genotype (1672 ± 465)]. However, for the *ALOX5AP* rs4769060 SNP, the effect on CorCP appears more consistent with additivity, with the 1/1 genotype having least CorCP (996 ± 114), the 2/1 genotype having medium CorCP (1244 ± 132), and the 2/2 genotype having the greatest CorCP (1404 ± 247).

For CarCP, the *ALOX5AP* rs10507391 SNP exhibited an additive pattern. The 1/1 genotype had greatest CarCP (360 ± 37), the 2/1 genotype had intermediate CarCP (313 ± 36), and the 2/2 genotype had the least CarCP (216 ± 43). The effect of the *ALOX5* rs3780906 SNP on IMT was also "additive," with the 1/1 genotype having the least IMT (0.666 ± 0.006), the 2/1 genotype having intermediate IMT (0.675 ± 0.007), and the 2/2 genotype having the greatest IMT (0.687 ± 0.016).

3.8. Functional Support for SNP Effects. The associations of lipoxygenase pathway gene variants (*ALOX* SNPs) with measures of atherosclerosis are indirect. The potential roles of these SNPs on mechanisms of atherosclerosis were explored by estimating the effect of each associated SNP in a select panel of biomarkers. The biomarkers evaluated in this population include adiponectin, leptin, ICAM-1, VCAM-1, E-selectin IL-6, CRP, MGP, and MCP-1 (Table 5).

Four lipoxygenase pathway variants contributed to variation in CorCP—one in *ALOX5* (rs2115819), one in

Table 3: Significance of association tests of *ALOX12*, *ALOX15*, *ALOX5*, and *ALOX5AP* SNPs with measures of atherosclerosis, independent of epidemiologic risk factors.

			P-values*			
Gene	LD Block	SNP	CorCP	CarCP	AorCP	IMT
ALOX12		rs9904779	0.308	0.853	0.533	0.874
(1 block)	1	rs2292350	0.523	0.552	0.436	0.827
		rs2271316	0.061	0.839	0.555	0.683
ALOX15		rs11568061	0.654	0.334	0.142	0.415
(2 blocks)	2	rs2515889	0.628	0.413	0.371	0.245
	1	rs2619112	0.872	0.117	0.425	0.296
ALOX5	1	rs745986	0.183	0.743	0.157	0.478
(8 blocks)	2	rs2115819	0.09	0.553	0.108	0.286
	3	rs892691	0.67	0.192	0.74	0.849
	4	rs3780906	0.603	0.203	0.541	0.09
	7	rs2291427	0.114	0.964	0.423	0.336
ALOX5AP		rs17244974	0.251	0.572	0.492	0.173
(5 blocks)	1	rs4769055	0.201	0.805	0.242	0.376
	2	rs10507391	0.288	0.085	0.648	0.473
	3	rs9551960	0.591	0.136	0.174	0.591
	4	rs9506352	0.097	0.369	0.188	0.511
	5	rs4769060	0.073	0.378	0.456	0.299

*Analyses performed using the quantitative pedigree disequilibrium test (QPDT); *P*-values adjusted for age, gender, diabetes status, smoking status, lipid lowering medication use, BMI, estrogen use, aspirin use, and hypertension medication use.

ALOX12 (rs2271316), and two in *ALOX5AP* (rs9506352 and rs4769060). The two *ALOX5AP* SNPs exhibited significant associations with levels of adiponectin (rs9506352, $P = .044$; rs4769060, $P = .007$), CRP (rs9506352, $P = .028$; rs4769060, $P = .002$); and MGP (rs9506352, $P = .003$; rs4769060, $P = .037$). Levels of MGP varied in a manner consistent with an additive gene effect, based upon the observed genotypic means [rs9506352 GG (9.06), GA (8.08), AA (7.64); rs4769060 AA (8.89), AG (8.41), GG (7.88)]. The *ALOX12* SNP also was associated with CRP level ($P = .036$), but also significantly associated with ICAM-1 level ($P = .032$) in a recessive pattern [rs2271316 GG (295.7), GC (268.3), CC (266.0)]. The *ALOX5* SNP (rs2115819) was associated with both CorCP and AorCP. This SNP was also associated with ICAM-1 ($P = .037$) and E-selectin ($P = .0001$); however, the genotypic means did not fit a classical single gene model, as the levels of both ICAM-1 and E-selectin for the heterozygote (T/C) class were greater than the means for the homozygote classes. Nonetheless, these data suggest that lipoxygenase pathway variants that are associated with CorCP affect markers of inflammation (CRP, ICAM-1) as well as arterial calcification (MGP).

One SNP in *ALOX5AP* (rs10507391) was associated with CarCP in this population. The *ALOX5AP* SNP identified a three-SNP haplotype (rs10507391-rs9551960-rs9506352) that was highly associated with CarCP. Each of these three individual SNPs was significantly ($P < .05$) associated with MGP level [rs10507391 AA (7.87), AT (8.63), TT (9.83); rs9551960 GG (7.69), GA (8.56), AA (9.64); rs9506352 GG (9.06), GA (8.08), AA (7.64)] as was the haplotype.

A single SNP in *ALOX5* (rs3780906) was associated with variation in IMT, with a three-SNP haplotype (rs3780901-rs3780906-rs1059696) providing strongest evidence of association. No consistent pattern of association was evident with any biomarker for the single SNP or the three-SNP haplotype.

4. Discussion

Type 2 diabetes is a major risk factor for cardiovascular disease (CVD), whose clinical outcomes include myocardial infarction and ischemic stroke. The principle etiologic factor for CVD is atherosclerosis which is thought to be the result of a chronic inflammatory process within a vessel wall that precipitates a cascade of events, from establishment of a fatty streak lesion to plaque formation [20]. The inflammatory process is triggered, in part, by oxidized lipids, including those of the lipoxygenase pathway. Recent evidence has demonstrated that lipoxygenases have two basic functions, (a) membrane modification by peroxidation and (b) lipid mediator signaling by G protein-coupled receptors [21, 22].

In the mouse, regulation of 12/15-LO and its pathway components (12S-HETE and 13S-HODE) in the vessel wall modulates aortic monocyte/endothelial cell interactions [23, 24], which are key early events in vascular inflammation [25]. Thus, the 12/15-LO pathway primarily affects atherosclerosis through LDL oxidation. An alternative pathway involves biosynthesis of proinflammatory leukotrienes (e.g., LTB_4) by 5-LO (and 5-LO-activating protein, FLAP,

Table 4: Genotypic means (± SD) of lipoxygenase variants in *ALOX5*, *ALOX5AP,* and *ALOX12* with atherosclerosis phenotypes.

Gene	SNP	1/1	1/2	2/2
CorCP				
ALOX5	rs2115819	1007 ± 2088	1211 ± 2489	1540 ± 2893
		181	264	454
		(247)	(352)	(145)
ALOX12	rs2271316	1065 ± 1639	1109 ± 2299	1423 ± 3155
		308	223	262
		(134)	(341)	(208)
ALOX5AP	rs9506352	1101 ± 2142	1188 ± 2416	1672 ± 3721
		265	229	254
		(331)	(332)	(64)
	rs4769060	996 ± 1767	1244 ± 2565	1404 ± 3086
		242	264	161
		(238)	(377)	(130)
CarCP				
ALOX5AP	rs10507391	360 ± 702	313 ± 694	216 ± 405
		60	71	5
		(358)	(368)	(89)
AorCP				
ALOX5	rs2115819	3208 ± 4350	2908 ± 3791	5318 ± 5563
		1256	1338	3158
		(188)	(291)	(105)
IMT				
ALOX5	rs3780906	0.666 ± 0.127	0.675 ± 0.129	0.687 ± 0.139
		0.649	0.654	0.645
		(436)	(348)	(75)

*The mean phenotypic value for each genotype (1/1, 2/1, 2/2) is provided with the median and the number of individuals (*n*) with each genotype included in the analysis; numbers vary based upon those with genotypic and phenotypic data.

Table 5: Association of lipoxygenase variants in *ALOX5*, *ALOX5AP,* and *ALOX12* with mean levels of biomarker phenotypes.

Gene	SNP		ICAM-1	CRP	IL-6	E-selectin	Leptin	Adiponectin	MCP-1	MGP
ALOX5	rs2115819	TT	269.6	0.62	4.34	47.7	15.2	11.82	460.6	8.44
		TC	294.8	0.66	4.58	79.7	17.9	11.01	446.5	8.47
		CC	236.4	0.64	4.16	54.2	13.6	11.05	468.9	8.53
	rs3780906	GG	269.8	0.66	3.95	67.6	13.6	11.13	444.7	8.48
		GA	286.7	0.65	4.77	59.6	21.2	11.53	459.5	8.54
		AA	228.9	0.56	4.5	51.9	6.6	11.8	505.3	7.87
ALOX12	rs2271316	GG	295.7	0.43	3.14	58.4	25.5	12.33	439.4	8.5
	(17947)	GC	268.3	0.75	4.68	59.3	15.7	11.03	465.3	8.59
		CC	266	0.56	4.31	67.3	13.4	11.01	429.8	8.09
ALOX5AP	rs10507391	AA	272.6	0.67	4.96	57.6	20.5	11.46	450.8	7.87
	(4431)	AT	262.2	0.6	3.9	63.8	13	11.77	459.3	8.62
		TT	290.1	0.7	4.82	77.1	15.7	9.52	459	9.83
	rs9506352	GG	273.2	0.69	4.3	68.7	12.9	10.69	451.7	9.06
	(13151)	GA	267.4	0.55	4.32	59.8	15.4	11.93	457	8.08
		AA	281	0.76	4.22	58.8	26	12.35	472.3	7.64
	rs4769060	AA	290.6	0.71	4.76	70.2	12.6	9.88	466.7	8.89
	(30231)	AG	260.9	0.57	4.23	61.1	14.6	12.1	450.9	8.41
		GG	278.1	0.74	4.4	63.2	22.6	11.75	443.7	7.88

which transfers arachidonate to 5-LO), providing access to downstream leukotrienes binding to G protein-coupled receptors. Recently, it has been shown that 5-LO pathway components are highly expressed in arterial walls in patients with atherosclerosis of the carotid and coronary arteries and the aorta [26].

Leukocyte-specific expression of human 15-LO has also been shown to reduce inflammation and atherosclerosis in rabbits [27, 28], most likely due to the generation of lipoxins from the dual action of 5-LO and 15-LO on arachidonic acid substrate in the leukocyte [29–31]. Studies in mice in which 15-LO was selectively expressed in endothelium indicated that endothelial-specific overexpression of 15-LO accelerated progression of atherosclerosis [12]. One plausible explanation for these differences is that endothelial cells do not possess 5-LO and are unable to directly generate lipoxins or related anti-inflammatory eicosanoids. The cellular source of lipoxygenase may be a critical determinant of atherosclerosis; hence, lipoxygenase pathways, their components, and their genetic determinants may be central to understanding the development of human atherosclerosis and CVD risk.

Genetic factors have long been known to modulate risk of atherosclerosis and CVD [32, 33]. In studies enriched with diabetic individuals or those with CVD, the genetic contribution to variation in carotid artery IMT ranges from 42%–92% [14, 34, 35]. Significant genetic contribution to calcified plaque has also been observed, whether in the coronary arteries [36], the carotid arteries [37, 38], or the aorta [39].

In the Diabetes Heart Study (DHS), variation in quantitative measures of subclinical atherosclerosis (CorCP, CarCP, AorCP, and IMT) appears to be differentially influenced by variants in genes of the lipoxygenase pathway. SNPs in *ALOX5* are associated with variation in CorCP, AorCP, and IMT. SNPs in *ALOX5AP* are associated with variation in CorCP and CarCP, while SNPs in *ALOX12* are associated with variation in CorCP. These results are consistent with findings that have been emerging from cellular and mouse models of atherosclerosis. Enhanced LDL oxidation, IL12 production, and endothelial/monocyte interaction have been observed through manipulation of 12/15-LO [11, 25, 40, 41]. In mouse genetic studies, a region on mouse chromosome 6 (the site of 5-LO) was shown to be linked to atherosclerosis susceptibility [42, 43]. Later, it was demonstrated that the disruption of only one 5-LO allele significantly reduced the extent of atherosclerotic lesions at the aortic root in LDLR−/− mice [2].

Previously, an *ALOX5* variant, defined by the number of Sp1 binding motifs in the promoter, was shown to be associated with variation in IMT and CRP level (a marker of chronic inflammation) in a healthy population [3]. The promoter variant was also shown to interact with dietary intake of 5-LO substrates. The study population (Los Angeles Atherosclerosis Study), in addition to being "healthy," was composed of several ethnic groups (Hispanic subjects and smokers were oversampled). The DHS, on the other hand, consists of European-American and African-American families with at least two diabetic siblings, making direct comparisons difficult. In addition, characterization of variation in the *ALOX5* gene was different (number of promoter Sp1 binding motifs versus SNPs within LD blocks across the entire *ALOX5* gene). Despite these differences in design and genetic evaluation, both studies observed that polymorphisms in *ALOX5* were associated with variation in measures of atherosclerosis (IMT in both studies; CorCP and AorCP in DHS). Unlike the Los Angeles Atherosclerosis Study, we did not detect an association between SNPs in LD blocks of *ALOX5* on ultrasensitive CRP level (data not shown).

The mechanism for associations between genes of the lipoxygenase pathway and subclinical atherosclerosis is not clear, although it may involve chemotaxis and proliferation that is induced by the effects of leukotrienes B_4 (LTB_4) signaling in vascular smooth muscle cells. LTB_4 has been detected in human carotid artery, atherosclerotic plaques and is derived from the 5-LO metabolism (via *ALOX5AP* and through G-coupled protein receptors) of arachidonic acid [21, 44]. Recently, a variant of the gene encoding LTB_4 hydrolase (*LTA4H*), a protein in the same biological pathway as *ALOX5AP* has been shown to be associated with risk of myocardial infarction [45], further strengthening the case for a role of the lipoxygenase pathway on CVD risk.

These data suggest that there may be differential effects of genes in a common pathway on several vascular beds through diverse inflammatory mechanisms (based upon the effects of lipoxygenase pathway SNPs on subclinical atherosclerosis and on markers of inflammation (CRP, E-selectin, ICAM-1) and aspects of calcification. Recently, serum MGP levels were determined in 2 independent populations free of clinically apparent cardiovascular disease [46] and an association of circulating MGP with increasing Framingham CHD risk score was observed, as were associations of circulating MGP with HDL and other individual CHD risk factors. Further characterization of genes in the lipoxygenase pathway may provide important clues to prediction of CVD. Although variation in these genes may be associated with risk of atherosclerosis, they may also modulate the impact of other atherosclerotic risk factors (e.g., lipid levels) or factors that are independent of traditional risk factors. The current data suggest that knowledge of the genetic profile of a pathway (and, by extension, the interaction of components of the pathway) may improve the prediction of risk. The extent of improvement should be greater once the multilocus examination of the pathway components becomes feasible. In this manner, the genetic "biological network" [47] of atherosclerosis may become a reality.

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Clinical Study

Relevance of Serum Leptin and Leptin-Receptor Concentrations in Critically Ill Patients

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The adipocyte-derived cytokine leptin was implicated to link inflammation and metabolic alterations. We investigated the potential role of leptin components in critically ill patients, because systemic inflammation, insulin resistance, and hyperglycemia are common features of critical illness. Upon admission to Medical Intensive Care Unit (ICU), free leptin and soluble leptin-receptor serum concentrations were determined in 137 critically ill patients (95 with sepsis, 42 without sepsis) and 26 healthy controls. Serum leptin or leptin-receptor did not differ between patients or controls and were independent of sepsis. However, serum leptin was closely associated with obesity and diabetes and clearly correlated with markers of metabolism and liver function. Leptin-receptor was an unfavourable prognostic indicator, associated with mortality during three years follow-up. Our study indicates a functional role of leptin in the pathogenesis of severe illness and emphasizes the impact of complex metabolic alterations on the clinical outcome of critically ill patients.

1. Introduction

Hyperglycemia, glucose intolerance, and insulin resistance are common features of critically ill patients, especially in patients with sepsis or septic shock, even in those without preexisting diabetes mellitus [1–3]. In patients with obesity, metabolic syndrome, and type 2 diabetes, several adipocytokines have been identified that mediate agonistic and antagonistic effects on insulin resistance [4, 5]. A link between adipocytokines, inflammation, and systemic insulin resistance has been established in obese and diabetic patients [5]. In critically ill patients, little is known about the actions of the different adipokines, especially about their potential impact on insulin resistance.

Since its identification in 1994 leptin, a 16-kilodalton hormone, has been investigated for its role in signalling food intake, glucose homeostasis, and energy expenditure through hypothalamic pathways [6–8]. Circulating leptin levels directly reflect adipose tissue mass and recent nutritional status in noncritically ill individuals [9]. The mechanisms of leptin expression are unclear, possibly insulin-stimulated glucose metabolism and peroxysome proliferator-activated receptor gamma (PPARγ) are involved in adipocyte leptin induction [10, 11]. Leptin exerts its various actions on glucose metabolism and energy expenditure via binding to the leptin-receptor in the brain and peripheral tissues as pancreas, liver, adipose tissue, and in the immune system [12]. In clinical settings, free-circulating leptin as well as soluble leptin-receptors that form complexes with circulating leptin are used to understand the pathogenetic role of leptin in regulating the inflammatory-metabolic response [13]. Various animal and human studies have shown that administration of endotoxin, TNFα, and other cytokines as inducers of severe systemic inflammation result in a significant elevation of serum leptin concentrations [14, 15].

Our study investigated serum leptin and leptin-receptor serum concentrations in a large cohort of critically ill patients (septic and nonseptic patients) from a medical ICU in order

Table 1: Characteristics of the study population.

Parameter	All ICU patients	Sepsis	Nonsepsis
Number	137	95	42
Sex (number male/number female)	85/52	59/36	26/16
Sex (% male/female)	62/38	62/38	62/38
Age median (years) (range)	63 (18–81)	64 (20–81)	60 (18–79)
BMI median (kg/m^2) (range)	25.4 (15.3–59.5)	25.65 (15.3–59.5)	24.9 (17.5–37.4)
Days at ICU median (range)	8 (1–137)	10 (1–137)	6 (1–45)
Days in hospital median (range)	25 (2–151)	30 (2–151)	14 (2–85)
Death at ICU *n* (%)	41 (29.9)	30 (31.6)	11 (26.2)
Death during follow-up *n* (%)	71 (51.8)	49 (51.6)	22 (52.4)
C-reactive protein median (mg/dL) (range)	112 (5–230)	167 (5–230)	14.5 (5–164)
Procalcitonin median (μg/L) (range)	0.9 (0.1–207.5)	2.2 (0.1–207.5)	0.2 (0.1–36.5)
IL-6 median (ng/L) (range)	110 (2–1000)	170 (7.7–1000)	40.5 (2–1000)
IL-10 median (ng/L) (range)	16 (5–1500)	20 (5–1500)	5.9 (5–750)
Protein median (g/L) (range)	52.5 (21–77)	52 (21–77)	55.5 (31–73)
Prothrombin time median (%) (range)	73 (11–100)	75 (11–100)	69 (13–100)
Creatinine median (mg/dL) (range)	1.6 (0.1–13.1)	1.9 (0.1–10.7)	1.2 (0.3–13.1)
Cystatin C median (mg/L) (range)	1.76 (0.41–7.30)	1.89 (0.41–6.33)	1.34 (0.41–7.30)
Glucose median (mg/dL) (range)	134 (47–663)	126 (47–299)	155 (65–663)
Insulin median (mU/L) (range)	9.8 (0.2–1000)	7.7 (0.2–438.0)	25.0 (0.2–1000)
C-peptide median (nmol/L) (range)	1.66 (0–13.0)	1.56 (0–13.0)	2.01 (0–11.6)
APACHE II score median (range)	14 (0–31)	14 (0–31)	15 (0–28)
SAPS-2 score median (range)	43 (0–80)	43 (0–79)	41.5 (13–80)

BMI: body mass index; IL: interleukin; ICU: intensive care unit; APACHE: Acute Physiology and Chronic Health Evaluation.

to understand the potential involvement of leptin and leptin-receptor in the pathogenesis of insulin resistance in critical illness, its regulation in severe systemic inflammation, and its potential clinical use as a biomarker in ICU patients.

2. Patients and Methods

2.1. Study Design and Patient Characteristics. The study was approved by the local ethics committee. Written informed consent was obtained from the patient, his or her spouse, or the appointed legal guardian. A total of 137 patients (85 male, 52 female with a median age of 63 years; range 18–81 years) was studied (Table 1). Patients were included consecutively upon admission to the ICU, if they were admitted to the Medical ICU of the University Hospital Aachen due to critical illness. Patients were not included in this study, if they were expected to have a short-term (<72 hours) intensive care treatment due to postinterventional observation or acute intoxication. Patient data, clinical information, and blood samples were collected prospectively.

The control group consisted of 26 healthy nondiabetic blood donors (17 male, 9 female, with a median age of 56 years; range 24–66 years) with normal values for blood counts, C-reactive protein, and liver enzymes.

2.2. Characteristics of Sepsis and Nonsepsis Patients. Among the 137 critically ill patients enrolled in this study, 95 patients conformed to the criteria of bacterial sepsis, according to the criteria of the American College of Chest Physicians & the Society of Critical Care Medicine Consensus Conference Committee for severe sepsis and septic shock [16]. In the majority of sepsis patients, the identified origin of infection was pneumonia ($n = 54$). Non-sepsis patients did not differ in age or sex from sepsis patients and were admitted to the ICU due to cardiopulmonary disorders (myocardial infarction, pulmonary embolism, and cardiac pulmonary edema; $n = 17$), decompensated liver cirrhosis ($n = 14$), or other critical conditions ($n = 11$). In sepsis patients, significantly higher levels of laboratory indicators of inflammation (i.e., C-reactive protein, procalcitonin, white blood cell count) were found than in non-sepsis patients (Table 1, and data not shown). Nevertheless, both groups did not differ in APACHE II score, vasopressor demand, or laboratory parameters indicating liver or renal dysfunction (data not shown). Among all critical care patients, 29.9% died at the ICU and 51.8% of the total initial cohort died during the overall follow-up of 900 days (Table 1). In sepsis and non-sepsis patients, no significant differences in rates of death and survival were observed (data not shown).

2.3. Comparative Variables. The patients in the sepsis and non-sepsis groups were compared by age, sex, body mass index (BMI), preexisting diabetes mellitus, and severity of disease using the APACHE II score at admittance. Intensive care treatment like volume therapy, vasopressor infusions, demand of ventilation and ventilation hours, antibiotic and antimycotic therapy, renal replacement therapy, and nutrition were recorded, alongside a large number of laboratory

parameters that were routinely assessed during intensive care treatment.

2.4. Quantification of Human Leptin and Leptin-Receptor. Human leptin serum concentrations were determined with a commercial ELISA (Cat. No. RD191001100, Bio Vendor). Intraassay (interassay) coefficient of variation (CV) ranged from 4.2% to 7.6% ($n = 8$) (4.4%–6.7% ($n = 6$)). Human leptin-receptor concentrations in serum were determined using a commercially available ELISA (Cat. No. RD194002100, Bio Vendor, Candler, NC). Intraassay (interassay) coefficient of variation (CV) ranged from 7.1% to 7.3% ($n = 8$) (6.2%–9.8% ($n = 6$)).

2.5. Statistical Analysis. Due to the skewed distribution of most of the parameters, data are given as median, minimum, maximum, 95% confidence interval. Differences between two groups are assessed by Mann-Whitney-*U*-test and multiple comparisons between more than two groups have been conducted by Kruskal-Wallis-ANOVA and Mann-Whitney-*U*-test for post hoc analysis. Box plot graphics illustrate comparisons between subgroups. They display a statistical summary of the median, quartiles, range, and extreme values. The whiskers extend from the minimum to the maximum value excluding outside and far out values which are displayed as separate points. An outside value (indicated by an open circle) is defined as a value that is smaller than the lower quartile minus 1.5-times interquartile range, or larger than the upper quartile plus 1.5-times the interquartile range. A far out value is defined as a value that is smaller than the lower quartile minus three times interquartile range, or larger than the upper quartile plus three times the interquartile range. All values, including "outliers", have been included for statistical analyses. Correlations between variables have been analysed using the Spearman correlation tests, where values of $P < .05$ were considered statistically significant. The prognostic value of the variables was tested by univariate and multivariate analysis in the Cox regression model. Kaplan Meier curves were plotted to display the impact on survival. All statistical analyses were performed with SPSS version 12.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Leptin and Leptin-Receptor Do Not Differ in Patients with Critical Illness from Healthy Controls. It had been reported that circulating leptin levels were low in critically ill patients upon admission to the ICU, possibly due to an acute stress response, with lowest levels in patients with sepsis [17]. We therefore tested free leptin and soluble leptin-receptor serum concentrations in 137 critically ill patients upon admission to our Medical ICU. Surprisingly, we did not observe significant differences in ICU patients as compared to healthy controls (Figure 1). Moreover, when we compared patients with sepsis ($n = 95$) and patients without sepsis ($n = 42$), no significant difference for either leptin or leptin-receptor concentrations could be detected (Figures 1(b) and 1(d)). Additionally, we performed subgroup analyses comparing patients with sepsis of pulmonary origin to those with abdominal or other septic focuses. However, leptin and leptin-receptor serum concentrations did not differ between these subgroups (data not shown).

Table 2: Correlation analysis.

	Leptin		Leptin-receptor	
	r	*P*	*r*	*P*
Inflammation				
Procalcitonin	−0.179	.043	0.252	.004
Interleukin-10	−0.190	.049	—	n.s.
Liver synthesis capacity				
Protein concentration	0.342	<.001	—	n.s.
Albumin concentration	0.295	.001	—	n.s.
Pseudocholinesterase	0.281	.001	−0.217	.012
Prothrombin time (%)	0.266	.002	—	n.s.
Antithrombin III	0.234	.012	−0.22	.017
Cholestasis				
*γ*GT	—	n.s.	0.204	.017
AP	—	n.s.	0.345	<.001
Bilirubin (total)	—	n.s.	0.298	<.001
Bilirubin (conjugated)	—	n.s.	0.373	<.001
Glucose metabolism				
Insulin	0.430	<.001	−0.294	.001
C-peptide	0.285	.001	—	n.s.
Lipid metabolism				
Cholesterol	0.216	.013	—	n.s.
HDL cholesterol	0.269	.003	—	n.s.
LDL cholesterol	0.270	.003	—	n.s.
Adipocytokines				
Adiponectin	−0.223	.015	0.548	<.001
Leptin	—	—	−0.587	<.001

*γ*GT: gamma-glutamyltranspeptidase; AP: alkaline phosphatase; HDL: high-density lipoprotein; LDL: low-density lipoprotein.

3.2. Leptin and Leptin-Receptor Are Associated with Obesity and Diabetes in Critically Ill Patients. Leptin is a cytokine mainly derived from adipose tissue and its concentrations directly reflect adipose tissue mass and the current nutritional status in noncritically ill individuals [4]. Consequently, we observed a close correlation between the patients' body-mass indices (BMI) and their leptin serum concentrations ($r = 0.478$, $P < .001$, Spearman rank correlation test; Figure 2(a)). By grouping the patient cohort to different BMI classes (<20, 20–25, 25–30, 30–35, >35), a clear increase in serum leptin with the BMI can be demonstrated (Figure 2(b)). In line, an inverse association between BMI and the circulating leptin-receptor could be revealed, with lowest leptin-receptor concentrations in obese patients with a BMI >35 kg/m^2 (Figures 2(c) and 2(d)).

Patients with a preexisting diabetes ($n = 46$) had significantly higher serum leptin levels (median 8.8 ng/mL) than patients without diabetes ($n = 91$, median leptin 4.9 ng/mL, $P = .013$; Figure 2(e)). However, leptin-receptor serum

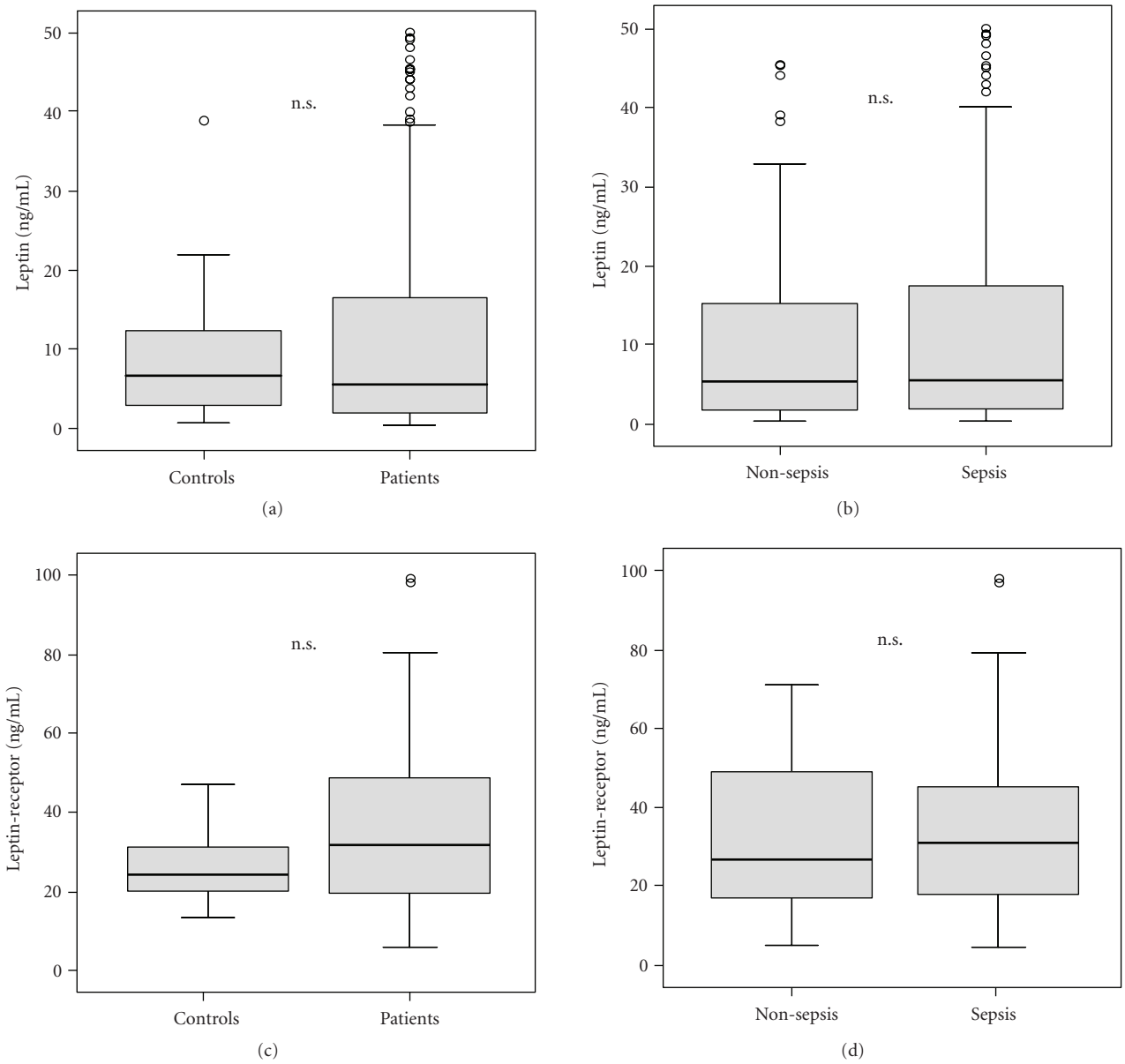

Figure 1: Serum leptin and leptin-receptor concentrations in critically ill patients. (a) Serum leptin levels are not different in critically ill patients ($n = 137$, median 5.5 ng/mL, range 0.4–49.6) as compared to healthy controls ($n = 26$, median 6.6 ng/mL, range 0.7–38.6). (b) No significant differences are detected between ICU patients with sepsis ($n = 95$, median 5.5 ng/mL, range 0.4–49.6) and nonseptic etiology ($n = 42$, range 5.4 ng/mL, range 0.4–45.1) of critical illness. (c) Leptin-receptor serum concentrations do not differ between critically ill patients (median 31.5 ng/mL, range 5.7–126.9) and healthy controls (median 23.9 ng/mL, range 13.1–46.4). (d) No difference in serum leptin-receptor concentrations can be observed between patients with (median 31.8 ng/mL, range 5.7–126.9) or without sepsis (median 29.0 ng/mL, range 7.4–124.7) at admission to the ICU. Box plot are displayed, where the bold line indicates the median per group, the box represents 50% of the values, and horizontal lines show minimum and maximum values of the calculated nonoutlier values; asterixes and open circles indicate outlier values.

concentrations did not differ in critically ill patients with or without diabetes (Figure 2(f)).

3.3. Leptin Is Correlated to Procalcitonin and Liver Dysfunction in Critical Illness. Leptin has been functionally linked to inflammatory proteins as it may directly interact with C-reactive protein [18]. We observed an inverse correlation between leptin and procalcitonin, but not to C-reactive protein or interleukin-6 (Table 2). With respect to organ dysfunction in the critically ill patients, we could not detect a significant correlation between leptin and renal function (e.g., glomerular filtration rate, cystatin C, creatinine; data not shown). However, the hepatic biosynthetic capacity was closely associated with serum leptin concentrations,

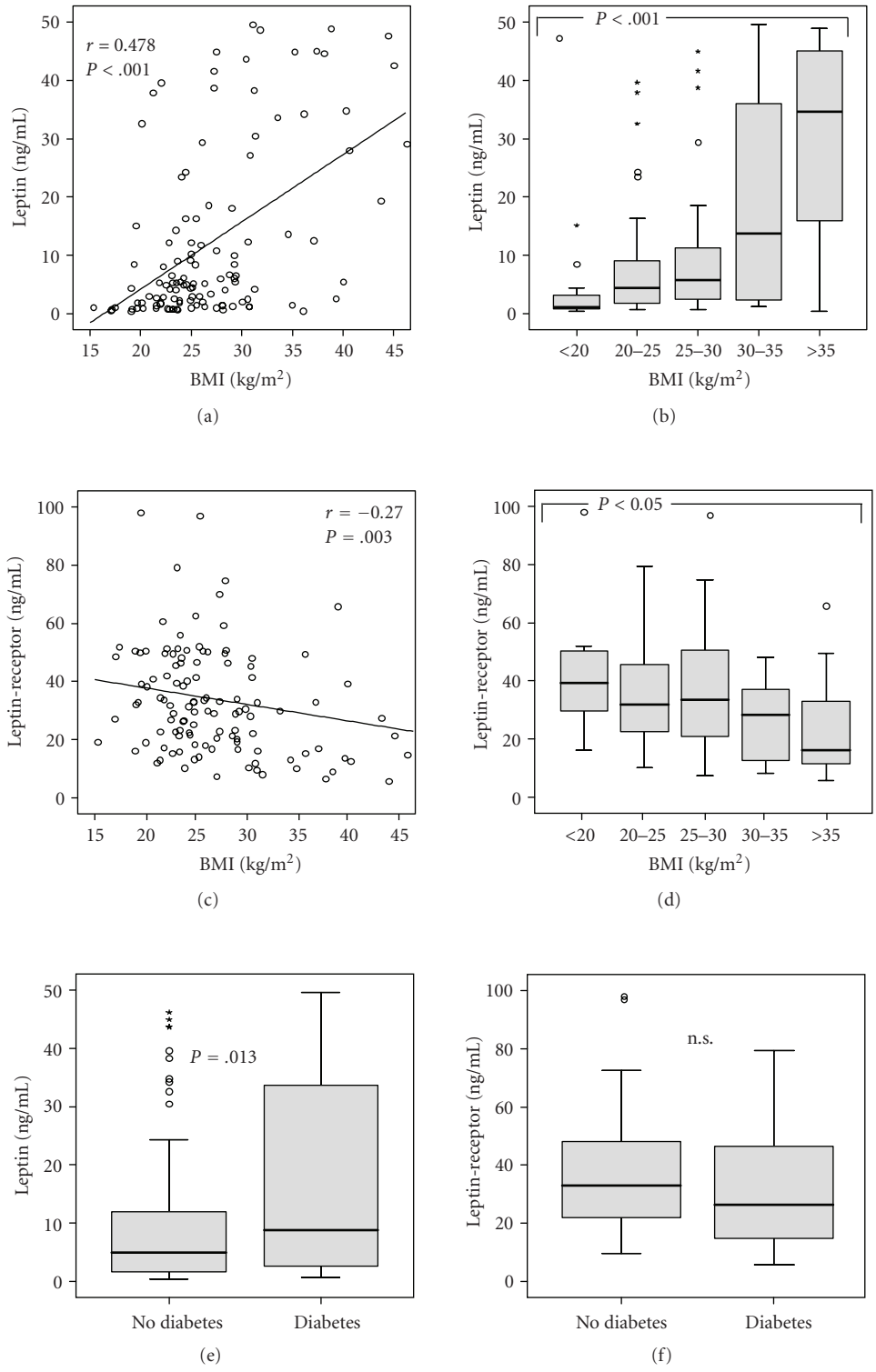

Figure 2: Association of serum leptin and leptin-receptor with obesity and diabetes in critically ill patients. (a) Serum leptin concentrations in ICU patients are correlated with the body-mass index (BMI). Spearman rank correlation test, correlation coefficient r, and P-values are given. (b) Among critically ill patients, serum leptin levels are significantly associated with the patient's BMI. Box-plot graphics are displayed for different classes of BMI; Kruskal-Wallis-test is used to assess significance of the differences. (c, d) In line, an inverse association between patient's BMI or obesity and serum leptin-receptor levels can be detected. (e) Serum leptin is significantly elevated in critically ill patients with preexisting diabetes in comparison to patients without diabetes. (f) Serum leptin-receptor concentrations do not differ in critically ill patients with or without diabetes. P-values (U-test) are given in the figure. Box plot are displayed, where the bold line indicates the median per group, the box represents 50% of the values, and horizontal lines show minimum and maximum values of the calculated nonoutlier values; asterixes and open circles indicate outlier values.

as evidenced by significant correlations to serum protein, albumin, prothrombin time, antithrombin III, and pseudocholinesterase activity (Table 2).

3.4. Leptin Is Associated with Glucose and Lipid Metabolism. Several observations in mouse models suggested that the actions of leptin on glucose homeostasis are independent of its effects on food intake, but associated with proper function of hypothalamic leptin-receptors [19, 20]. We observed a close correlation of insulin and C-peptide with leptin and an inverse correlation with leptin-receptor (Table 2). Furthermore, markers of lipid metabolism, for example, total cholesterol, HDL cholesterol, and LDL cholesterol were closely associated with leptin serum concentrations (Table 2).

3.5. Leptin-Receptor Is Not Linked to Metabolic Parameters, but to Procalcitonin and Parameters Reflecting Cholestasis. In contrast to leptin, a correlation between serum leptin-receptor concentrations and metabolic parameters could not be found (Table 2). An association between leptin-receptor and procalcitonin as well as insulin could be observed, but not with other inflammatory or metabolic parameters (Table 2, and data not shown). Interestingly, leptin-receptor showed significant correlations to various parameters reflecting cholestasis such as bilirubin, conjugated bilirubin, gamma-glutamyltranspeptidase, and alkaline phosphatase activity (Table 2). This might indicate that biliary excretion could be possibly involved in leptin-receptor clearance.

3.6. High Leptin-Receptor Levels Indicate Unfavourable Prognosis in Critically Ill Patients. Cox regression analyses and Kaplan-Meier curves were used to assess the impact of leptin and leptin-receptor on ICU and overall survival during a nearly three-year follow-up among critically ill patients. No significant association between leptin and the ICU- or overall-survival of the patients could be identified using uni- and multivariate Cox regression analysis (data not shown). Remarkably, leptin-receptor serum concentrations upon admission to the Medical ICU were an unfavourable indicator of ICU survival ($P = .047$) as well as of overall survival ($P = .034$, Cox regression analysis). Using a cut-off value for leptin-receptor of 32 ng/mL, Kaplan-Meier curves were plotted to display mortality (log rank 6.77; Figure 3(a)). In line, surviving patients had significantly lower leptin-receptor concentrations (median 26.8 ng/mL) than nonsurvivors (median 34.5 ng/mL, $P = .037$, U-test; Figure 3(b)).

4. Discussion

Hyperglycemia and insulin resistance are common in critically ill patients and have been identified as adverse prognostic predictors in ICU patients. In obesity and type 2 diabetes, adipocytokines are critical mediators linking chronic inflammatory conditions to systemic insulin resistance [5]. The role of adipocytokines in sepsis and nonseptic ICU patients is currently unclear. The best studied adipocytokine in critical illness at present is resistin, which was suggested to serve as an acute-phase component as it is strongly upregulated in patients with severe sepsis and septic shock [21, 22]. Very little is known about the potential role of leptin in this clinical condition.

In a recently published study, it has been reported that serum leptin concentrations are low in all critically ill patients on admission to the ICU, with lowest levels in sepsis patients [17]. As a possible mechanism, reduced synthesis or increased removal either by extravasation due to capillary leakage in sepsis or increased metabolic clearance has been speculated [17]. In this study by Langouche et al., the SOFA score on admission was statistically significantly higher in sepsis patients as compared to nonseptic patients. Moreover, a prior study demonstrated decreased plasma leptin levels due to trauma which were explained to be partly related to the initial fasting conditions, because refeeding elevated serum leptin concentrations to normal levels [23].

In our study, the leptin and leptin-receptor concentrations did not differ in patients with critical illness from healthy controls. Subgroup analysis revealed no significant difference in sepsis and non-sepsis patients as well (Figure 1). This difference might very likely be related to our cohorts of septic and nonseptic patients, which were very homogenous in terms of severity of illness as reflected by APACHE II and SOFA score. Moreover, our study population had a slightly higher median BMI and therefore possibly a better nutritional status in the subgroups of patients with and without proven sepsis. However, in contrast to prior observations, our data indicate that circulating leptin and leptin-receptor levels are not severely dysregulated in critically ill patients at the point of admission to the ICU.

Leptin has various peripheral and central targets, including cells of the brain, pancreas, liver, adipose tissue, and immune system [12]. In animal models, deletion of the cerebral leptin-receptor leads to obesity and elevated plasma levels of leptin, glucose, and insulin [24]. Leptin deficiency in humans either due to an absolute shortage of leptin or due to leptin-receptor mutations causes severe early onset obesity, hyperphagia, hyperinsulinemia, hypogonadism, and impaired T cell function [25]. In individuals with leptin-receptor defects, the features of leptin deficiency appear ameliorated, which might give a hint for the existence of alternative, leptin-receptor independent, leptin signalling pathways [26]. In line with the current view of leptin as being a cytokine, mainly derived from adipose tissue and reflects adipose tissue mass, we found a close association to the patients' BMI (Figures 2(a) and 2(b)). Accordingly, an inverse association could be observed for the circulating leptin-receptor (Figures 2(c) and 2(d)), which is expressed by various tissues and serves as a binding partner for leptin in the circulation [24].

The regulation of glucose metabolism by leptin is likely independent of its effect on food intake and energy expenditure. Hypothalamic expression of leptin-receptor in animal model resulted in modest reduction of food intake and body fat mass, but in normalized blood glucose and insulin levels [19]. The present data suggest the existence of two leptin signalling pathways: the leptin-receptor mediated JAK-STAT (Janus kinase signal transducers and activators

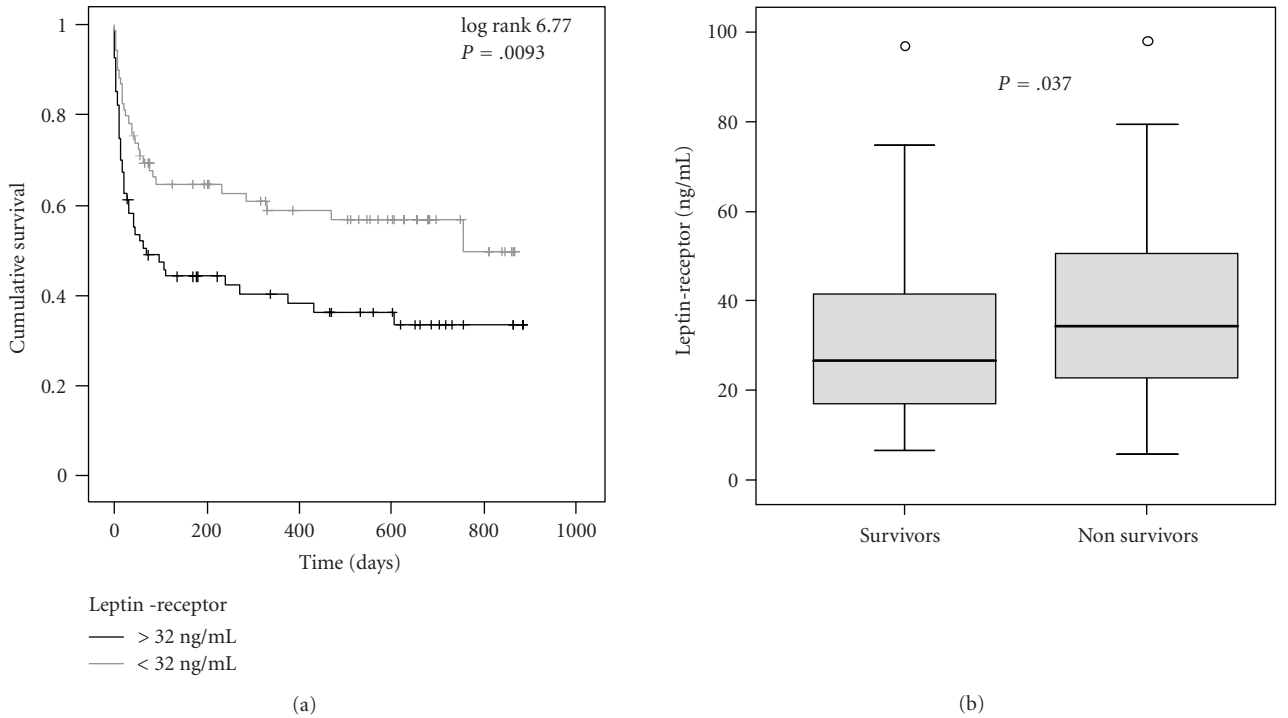

Figure 3: Prognostic relevance of serum leptin-receptor in critically ill patients. (a) Kaplan-Meier survival curves of ICU patients ($n = 137$) are displayed, showing that patients with high leptin-receptor levels (>32 ng/m/L, black) have an increased mortality as compared to patients with low serum leptin-receptor (≤32 ng/mL, grey). P-value from Cox regression analysis is .034. (b) Patients that die during follow-up period treatment ($n = 71$, 51.8%) have significantly ($P = .037$) higher serum leptin-receptor levels on admittance to ICU than survivors ($n = 66$, 48.2%). Box plot are displayed, where the dotted line indicates the median per group, the box represents 50% of the values, and horizontal lines show minimum and maximum values of the calculated nonoutlier values; asterixes and open circles indicate outlier values.

of transcription) signalling for regulation of food intake and body weight, and PI3K (phosphoinositide-3 kinase) pathway for regulation of glucose metabolism [19, 20, 27]. These experimental findings might very well explain why we did not only observe a strong association between patients' BMI and leptin, but also between glucose metabolism (e.g., insulin secretion) and serum leptin levels in critically ill patients.

The possible interaction between leptin and systemic inflammation is a matter of ongoing debate. It has been reported that human C-reactive protein (CRP) inhibits the binding of leptin to its specific receptor and blocks signal transduction in cultured cells and mouse models; thus, it might attenuate the physiological function of leptin and contribute to the concept of "leptin resistance" [18]. In our study, we could demonstrate an inverse correlation between leptin and procalcitonin and a direct correlation between leptin-receptor and procalcitonin, but not with "classical" markers of inflammation as C-reactive protein or interleukin-6. Possibly, acute bacterial inflammation, as displayed by procalcitonin, in critically ill patients contributes to the state of "leptin resistance," yet the clinical impact of "leptin resistance" in critical illness is still unsettled.

Furthermore, we analyzed the association of leptin with markers of organ function in ICU patients. Leptin did not correlate with renal function as reflected by glomerular filtration rate, cystatin C, and creatinine. We observed that the hepatic biosynthetic capacity was closely related to leptin levels (Table 2). This finding in critically ill patients is in contrast to reports from patients with liver cirrhosis, in which leptin levels are rather upregulated [13, 28].

Remarkably, leptin-receptor serum concentrations upon admission to the Medical ICU were an unfavourable indicator of ICU survival ($P = .047$) as well as of overall survival ($P = .034$) (Figures 3(a) and 3(b)). Surviving patients had significantly lower leptin-receptor concentrations than nonsurvivors. This observation was highly unexpected, especially since leptin-receptor was not an obvious surrogate marker for other alterations, for example, inflammation, organ failure, in critically ill patients. The association with survival was independent from other parameters by multivariate regression analysis (data not shown). The exact functional contribution of soluble leptin-receptor or free leptin in the pathogenesis of critical illness or sepsis is currently unclear, and additional studies in experimental models are warranted. It is also important to note that future studies should incorporate longitudinal measurements of leptin and leptin receptor during the course of critical illness, as this might help to clarify the potential pathogenic contributions at different phases of initiation and recovery from acute illness. However, the association of leptin-receptor with survival raises the possibility that including serum leptin-receptor

concentrations might improve the validity of prognostic assessments in critically ill patients upon admission to the ICU.

5. Conclusions

Although leptin and leptin-receptor serum concentrations do not differ in patients with critical illness or in the subgroups of patients with and without sepsis from healthy controls, serum leptin in critically ill patients is closely correlated with the patients' BMI and metabolic alterations. The possible functional role of leptin in the pathogenesis of severe illness warrants further studies. However, soluble leptin-receptor turned out to be an independent prognostic marker at admission to the Medical ICU, thereby emphasizing the impact of the complex metabolic alterations on the clinical outcome of critically ill patients.

Acknowledgments

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Review Article

Inflammatory Mediators and Insulin Resistance in Obesity: Role of Nuclear Receptor Signaling in Macrophages

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Visceral obesity is coupled to a general low-grade chronic inflammatory state characterized by macrophage activation and inflammatory cytokine production, leading to insulin resistance (IR). The balance between proinflammatory M1 and antiinflammatory M2 macrophage phenotypes within visceral adipose tissue appears to be crucially involved in the development of obesity-associated IR and consequent metabolic abnormalities. The ligand-dependent transcription factors peroxisome proliferator activated receptors (PPARs) have recently been implicated in the determination of the M1/M2 phenotype. Liver X receptors (LXRs), which form another subgroup of the nuclear receptor superfamily, are also important regulators of proinflammatory cytokine production in macrophages. Disregulation of macrophage-mediated inflammation by PPARs and LXRs therefore underlies the development of IR. This review summarizes the role of PPAR and LXR signaling in macrophages and current knowledge about the impact of these actions in the manifestation of IR and obesity comorbidities such as liver steatosis and diabetic osteopenia.

1. Introduction

Progressive development of insulin resistance (IR) is a prediabetic state which is today a widespread metabolic abnormality of adults and adolescents in industrialised societies [1]. Impaired insulin action is considered the first stage of type 2 diabetes mellitus (T2DM). The consequences of IR manifest at many levels and in many metabolic processes, producing a cluster of homeostatic abnormalities including glucose intolerance, overt hyperglycemia, hyperinsulinemia, and atherogenic dyslipidemia, collectively referred to as metabolic syndrome (MetS). Liver steatosis, kidney disease, and osteoporosis are also frequent comorbidities of T2DM and MetS [2–4].

IR correlates positively with obesity, and the rapidly growing incidence of T2DM and MetS is therefore often attributed to lifestyle factors such as excess caloric intake and insufficient physical exercise in urbanized human populations [5]. The main predisposing factor for IR is intra-abdominal accumulation of adipose tissue (AT), which leads to central obesity [5, 6]. The total load of visceral adipose tissue (VAT) and the rate of free fatty acid (FFA) mobilization from VAT to the portal venous system are well-established correlates of IR and high circulating levels of insulin [7–9]. Several mechanisms link visceral adiposity and elevated FFA levels to IR. The elevated VAT mass liberates excess amount FFAs to the bloodstream, which contribute to muscle and liver IR by triggering reduced insulin signaling and increased hepatic gluconeogenesis. High levels of FFA shift the substrate preference of mitochondrial oxidation from glucose to FFA, and this can diminish the insulin secretory response to glucose of islet β-cells, leading to relative insulin insufficiency [10, 11]. Moreover, FFAs induce an inflammatory response in macrophages, adipocytes, and muscle cells via toll-like receptor (TLR) activated pathways (Figure 1). Modified lipoproteins such as oxidized and glycated low-density lipoproteins derived from excess VAT can accumulate in certain tissues, including subendothelial spaces, muscle cells, liver or kidney mesangium and tubular epithelial cells, where they can give rise to atherogenesis, lipotoxic injury,

and inflammation [12]. VAT is also an active endocrine organ able to secrete a wide variety of inflammatory cytokines with key functions in the development of IR [13].

In recent years, macrophages have been recognized as major sources of proinflammatory mediators, which are largely responsible for the manifestation of IR. Macrophages are plastic cells and their ability to produce cytokines is determined by their phenotype. The so-called classical activated or "M1" macrophages secrete high amounts of inflammatory mediators while the alternatively activated "M2" macrophages are low cytokine producers. In obesity the balance between M1 and M2 macrophages is disturbed. Thus, production of inflammatory cytokines by VAT macrophages increases significantly [14]. This situation creates a general subclinical inflammatory state [15] that will ultimately lead to altered insulin responsiveness. Recent studies reveal that macrophage activation is regulated by lipid metabolites through the activation of nuclear receptor transcription factors, and that imbalances in macrophage nuclear receptor signaling can lead to IR [13].

Nuclear receptors (NRs) are a superfamily of ligand-activated transcription factors that control transcription of their target genes through direct or indirect mechanisms. Directly, NRs bind to specific DNA sequences in *cis*-regulatory elements within promoter regions, activating or repressing target gene expression by recruiting or releasing coactivators and corepressors [16]. Indirectly, NRs can *trans*-repress the transcription of certain genes controlled by other transcription factors, such as nuclear factor kappa-B (NF-κB) or activator protein-1 (AP-1) [17, 18]. Prominent members of the NR superfamily are peroxisome proliferator activated receptors (PPARs), activated by FFAs, eicosanoids, and prostaglandins, and liver X receptors (LXRs), activated by cholesterol metabolites. These "lipid sensors" appear to play a central role in the control of lipid metabolism. NRs are moreover the targets of environmental obesogens such as phtalates, organotins, bisphenol A, and xenobiotics that interfere with NR signaling and which are thought to underlie the spread of obesity and its comorbidities [19]. In addition, evidence acquired over the last decade demonstrates that PPARs and LXRs have important antiinflammatory effects and can control macrophage activation, suggesting potential in the medication of IR.

The role of NRs in linking metabolism and inflammation is especially relevant to the pathogenesis of obesity-induced IR. Synthetic pharmacological ligands for PPARγ (thiazolidinediones; TZDs) and PPARα (fibrates) are used clinically due to their hypolipidemic and insulin-sensitizing properties. Additionally, pharmacological activation of LXRs results in increased HDL levels and net cholesterol loss, therefore, synthetic LXR ligands have a potential medical benefit to treat dyslipidaemias and atherosclerosis. A growing body of literature suggests that these drugs, due to their antiinflammatory effects, can have a broader impact in metabolic diseases, especially in obesity comorbidities. Here we summarize the latest findings linking IR, inflammatory mediators, and macrophages and discuss the regulatory role of NR signaling in macrophage cytokine production associated with obesity and obesity comorbidities.

2. Friend or Foe? M1 and M2 Macrophages in Adipose Tissue

Over the last few years, understanding of macrophages as an important element of IR development has advanced considerably with the identification of distinct functional macrophage subsets. Macrophages have a highly plastic phenotype that allows them to specialize and display polarized functional properties, such as inflammatory or antiinflammatory actions in response to cytokines and microbial products. Macrophage polarity can be determined by T-helper cells. Cytokines released by T-helper 1 (Th1) cells, such as interleukin-2 (IL-2), gamma-interferon (IFNγ), and tumor-necrosis factor alpha (TNFα), induce the classical macrophage phenotype, activating them to stimulate cellular immunity and inflammation. Th1 cells also secrete granulocyte-macrophage colony stimulating factor (GM-CSF), which promotes medullar monocyte/macrophage differentiation. In contrast, T-helper 2 (Th2) cells secrete interleukin-4 (IL-4) and interleukin-13 (IL-13), which induce an alternative phenotype by attenuating macrophage-mediated secretion of inflammatory mediators and instead inducing macrophage programs for FFA oxidation [20]. Adapting the Th1/Th2 nomenclature, Mantovani and colleagues in 2002 started to refer to polarized macrophages as M1 and M2 cells [21]. M1 macrophages are activated proinflammatory cells, while M2 macrophages are characterized by an antiinflammatory phenotype. Although there is a clear association of obesity and IR with macrophage infiltration of AT and M1 macrophage activation, the dominant phenotype of adipose tissue macrophages (ATMs) is still an open debate.

The first evidence suggesting diversity of ATM phenotype was obtained from chemokine receptor-2 (CCR2) knockout (KO) mice (CCR2KO) [22]. CCR2 is a cell-surface receptor for monocyte chemoattractant protein-1 (MCP-1), a chemokine which specifically mediates monocyte chemotaxis. Under normal physiological conditions, the ATM content of CCR2KO AT does not differ from wild-type AT, and CCR2KO mice show no overt metabolic alteration. However, CCR2KO mice fed a high-fat diet accumulate fewer ATMs in AT than similarly fed wild-type mice, and present an attenuated inflammatory profile and greater insulin sensitivity. Thus metabolic challenge with supernormal fat intake triggers macrophage recruitment to VAT via a MCP-1/CCR2-dependent process, but CCR2 is not required for resident macrophage recruitment. Brake and co-workers subsequently identified CD11c-positive ($CD11c^+$) and CD11c-negative ($CD11c^-$) macrophage populations in mouse AT [23]. The numbers of $CD11c^+$ cells increase in response to a high-fat diet, and this is accompanied by increased AT expression of transcripts for CCR2, interleukin-6 (IL-6), and intercellular adhesion molecule I (ICAM-I), a leukocyte adhesion receptor needed for macrophage tissue infiltration. $CD11c^+$ cells were thus proposed as an inflammatory macrophage

population in AT. Interestingly, conditional bone marrow depletion of $CD11c^+$ cells in obese mice results in a rapid normalization of insulin sensitivity [24]. Moreover, further studies confirmed that ATMs recruited to AT in diet-induced obesity express high levels of IL-6, inducible nitric oxide (NO) synthase (iNOS) and CCR2, all characteristics of the M1 phenotype [25, 26]. In obese mice chronic iNOS blockade attenuates high-fat diet induced IR and, similar to CCR2KO mice, reduces macrophage VAT infiltration, as shown by lowered mRNA expression of MCP-1 and the macrophage cell surface receptor CD68 [27]. In addition, resident ATMs show very low (if any) inflammatory activity and express many M2-associated genes such as arginase 1, interleukin-10 (IL-10) and the secretory chitinase protein Ym1 [28].

The identification of two monocyte populations in mouse blood [29, 30] supported the hypothesis that M2 macrophages resident in AT are descendents of circulating nonactivated monocytes, while M1 macrophages derive from a population of circulating inflammatory monocytes that are recruited to AT where they continue their differentiation and orchestrate the inflammatory response. This model is further supported by the observation that, blood mononuclear cells from obese patients are in a proinflammatory state [31].

An alternative hypothesis is that M1 macrophage polarization during obesity progression occurs via in situ reprogramming of ATMs from an M2 to an M1 phenotype. In vitro, it is well established that the pattern of macrophage function depends on the agonist to which they are exposed [32]. For instance, in vivo, treatment of tumor-bearing animals with interleukin-12 (IL-12) shifts tumor-associated macrophages from a dominant M2 profile (elevated expression of TGFβ, IL-10, and MCP1) to a proimmunogenic/inflammatory M1 profile (elevated expression of IL-6 and TNFα) [33]. However, it remains uncertain whether this "in situ" phenotype switching can also occur in AT (Figure 1).

Interestingly, PPARγ and PPARδ have been recently implicated in the transcriptional regulation of monocyte/macrophage phenotypic shift (Figure 1). Using myeloid-specific PPARγ and PPARδ KO mice (Mac-PPARγKO and Mac-PPARδKO), Odegaard et al. showed that PPARγ and PPARδ are both necessary for optimal induction of the M2 macrophage phenotype by IL-4 (a classical Th2 cytokine) [34, 35]. However, these factors make distinct contributions to this process: PPARγ is specifically required for IL-4-dependent activation of fatty acid oxidation, whereas PPARδ is required for the full expression of the IL-4-dependent immune phenotype (Figure 1). Furthermore, the AT of fat-fed Mac-PPARγKO accumulates fewer macrophages and shows lower M2-related gene expression than the AT of fat-fed wild-type mice. However, fat-fed Mac-PPARγKO mice are more obese, indicating that the reduced number of M2 macrophages leads to major alterations in adipocyte metabolism [34]. These studies demonstrate that activation of PPARγ and PPARδ in ATMs ameliorates IR not only through the regulation of cytokine production but also by modulating ATM phenotype.

3. Nuclear Receptor Signaling Reduces Cytokine Production by ATMs and Ameliorates Insulin Resistance

The paracrine and endocrine functions of VAT actively contribute to the development of IR. VAT is a major source of a wide variety of cytokines produced mainly by macrophages and of certain hormone-like factors produced by adipocytes. The best known VAT-produced cytokines include C-reactive protein (CRP), IL-6, interleukin-1 (IL-1), interleukin-18 (IL-18), and tumor necrosis factor (TNFα) [36]. These inflammatory mediators exert their actions not only on AT cells, but also on other cell types such as hepatocytes, liver Kuppfer cells, kidney mesangial cells, osteoclasts, and muscle fibers. Indeed, in T2DM patients, elevated VAT expression of TNFα is associated with the onset of IR, and high circulating levels of interleukin-1 receptor antagonist (IL-1ra) and TNFα correlate strongly with MetS in human populations [37]. The mechanisms by which inflammatory cytokines produce defects in insulin signaling are not fully understood; however, many studies suggest an origin in insulin postreceptor signaling. Binding of insulin to its receptor is followed by phosphorylation of the insulin receptor substrates IRS-1 and IRS-2. Tyrosine phosphorylation of IRS-1 and IRS-2 mediates insulin signaling; however, serine phosphorylation of IRSs can block downstream signaling. There are thus two pathways by which cytokines appear to interfere in insulin signaling: by impairing IRS tyrosine phosphorylation or by inducing IRS serine phosphorylation [38]. For instance, TNFα impairs tyrosine phosphorylation mediated by PI3-kinase, leading to insufficient glucose uptake by muscle cells [39, 40]. In addition, there is evidence implicating the serine kinases c-Jun Kinase (JNK) and inhibitor of NF-κB kinase (IKK) in cytokine-dependent IR: obesity is associated with increased JNK activity in adipose and liver tissues [41] and mice lacking IKK-β are resistant to obesity-induced IR [42]. These kinases also affect AP-1 and NF-κB transcription factors, promoting further inflammatory gene expression. In addition, SOCS proteins, another class of inflammatory mediators, have been found to be involved in obesity-induced IR. SOCS proteins block insulin signaling either by interfering with IRS-1 and IRS-2 phosphorylation or by targeting IRSs for proteosomal degradation [43, 44].

Studies on PPARs and LXRs indicate that these nuclear receptors are important regulators of proinflammatory cytokine production by macrophages. In LPS- or IFNγ-stimulated macrophages, activation of PPARγ represses the induction of inflammatory genes including iNOS, IL-6, cyclooxygenase-*2 (*COX-2), and matrix metalloproteinase 9 (MMP9) [45, 46]. Activation of LXRs represses almost the same genes as PPARγ [47, 48], while PPARα shows a distinct pharmacological profile, inhibiting expression of tissue factor [49]. Finally, PPARδ deficiency in macrophages is associated with low levels of MMP9 and MCP1 [50]. Recently, increased cytokine production has been reported in vivo in the AT, liver, and muscle of myeloid PPARγKO mice, correlating with the development of IR in these animals [51].

Most evidence indicates that the basic mechanism underlying the antiinflammatory actions of NRs is interference in

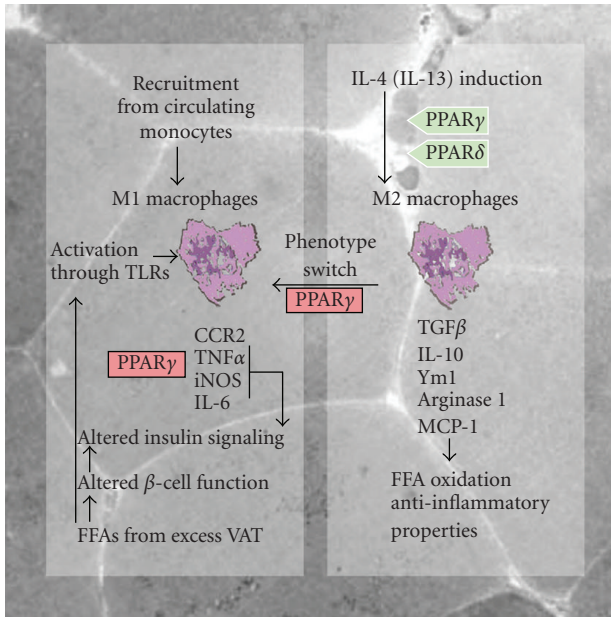


Figure 1: Cytokine release by adipose tissue macrophages contributes to insulin resistance. Free fatty acids (FFAs) released from visceral adipose tissue (VAT) promote polarization toward the M1 phenotype through activation of Toll-like receptors (TLRs), and also impair insulin secretion and action. Differentiation to the M1 phenotype is inhibited by PPARγ signaling, and activation of PPARγ or PPARδ, in response to IL-4, promotes polarization toward the M2 phenotype. IL-13 is also suggested to be involved in the M2 phenotype switch. Inflammatory monocytes migrating into VAT can also differentiate into M1 macrophages. Inflammatory mediators produced by M1 ATMs alter insulin responsiveness. CCR2: chemokine receptor-2, TNFα: tumor necrosis factor, iNOS: inducible nitric oxide synthase, IL-6: interleukin-6, TGF-β: transforming growth factor beta, IL-10: interleukin-10, Ym1: secretory chitinase protein-1, MCP-1: monocyte chemoattractant protein-1 (MCP-1).

AP-1/NFκB signalling [17, 18]. However, alternative pathways are not excluded. For example, mechanisms involving blockade of the clearance of corepressor complexes from promoters have recently been proposed. These processes are thought to involve SUMOylation of PPARγ or LXRs [52]. There thus appears to be no single mechanism of repression, and pathway selection seems to depend on the signal, the NR isoform involved, and even the gene promoter.

The ability of PPAR and LXR receptors to control macrophage-mediated inflammation by these mechanisms appears to have an important impact on the control of IR. Indeed, the beneficial effect of weight loss on obesity-related IR might be associated with an improved inflammatory profile in the stromal vascular fraction of AT, which contains the ATMs [53].

Unlike other AT related proteins, the adipocyte protein, adiponectin, contributes to the maintenance of insulin sensitivity and seems to be able to antagonize the proinflammatory effects of macrophages [54, 55]. Adiponectin is the most abundant adipocyte-derived factor in the circulation and low levels of this protein are linked to high body mass index, IR, dyslipidemia, and increased risk of cardiovascular disease [56]. Consistently, adiponectin immunostaining is reduced in the AT of mice fed a fat- and carbohydrate-rich diet [57]. In humans, a marked gender difference in AT distribution evolves during puberty, resulting in elevated VAT mass and lower adiponectin production in adult males and an associated higher susceptibility to insulin signaling defects [58, 59]. Moreover, TNFα reduces adiponectin production [60]. Importantly, adiponectin also has a potent antiinflammatory action on macrophages, suppressing lipopolysaccharide (LPS)-stimulated cytokine production possibly via the antiinflammatory IL-10 signaling pathway [61]. Adiponectin promoter is regulated by PPARγ/RXRα heterodimers, and administration of TZDs has been reported to significantly increase plasma adiponectin concentrations in insulin-resistant humans and rodents without affecting their body weight [62]. Activation of PPARγ induces production of adiponectin not only from adipocytes but also from skeletal muscle, which augments the antidiabetic actions of PPARγ [63, 64].

4. Liver Resident Macrophages Link Obesity to Steatosis

The liver is responsible for the coordination of intermediate metabolism. Hepatocytes are actively involved in glucose and lipid metabolism (including cholesterol and lipoprotein synthesis), plasma protein synthesis, and the production of inflammatory proteins such as CRP [65]. Obesity is associated with a high incidence of steatosis, a pathological accumulation of lipids within hepatocytes. Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease and is characterized by excess liver lipid

accumulation and hepatic IR. At a later stage of disease progression, NAFLD can occur with hepatic inflammation, leading to nonalcoholic steatohepatitis (NASH) and culminating in hepatic fibrosis or cirrhosis [3].

In obesity, inflammatory cytokines (IL-1β, TNFα, and IL-6) and adiponectin released from AT reach the liver through the portal vein and can directly interfere with liver functions. In an inflammatory state, TNFα can trigger hepatocyte apoptosis and the activation of the fibrogenic response in stellate cells [66], while IL-6 is implicated in the induction of the acute phase response by eliciting transcriptional activation of CRP [67]. Adiponectin, unlike cytokines, appears to have a protective effect in the liver. Adiponectin administration ameliorates steatosis, probably via inhibition of TNFα signaling. In addition, adiponectin exerts antifibrogenic effects: adiponectin KO mice exposed to CCL4 develop more severe fibrosis than wild-type animals [68].

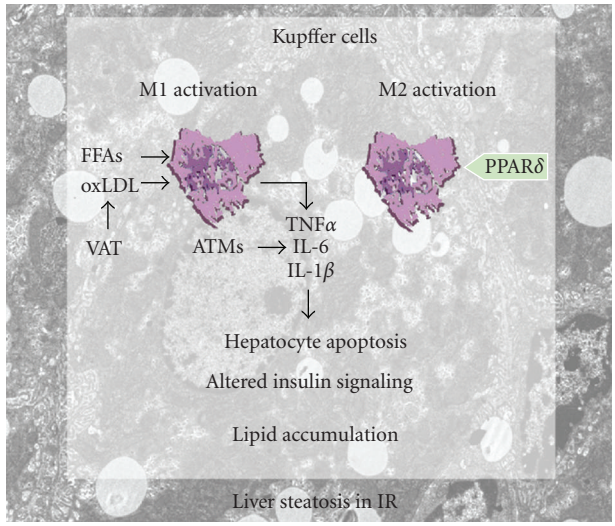
However, the most important sources of inflammatory cytokines within the liver are Kupffer cells, the resident macrophages in the liver. Kupffer cells mostly localize in the liver sinusoids but can also migrate into the space of Disse. Like all macrophages, they show phenotypic plasticity, presenting different morphology and functions depending on their intralobular position. Kupffer cells located in the periportal zone are large cells with high phagocytosis capacity and high lysosomal protease activity, whereas Kupffer cells in mid-zonal and perivenous areas are smaller and have lower protease activity [69, 70]. During steatosis, the recruitment of new macrophages into the liver can alter cell distribution, thereby also changing Kupffer cell morphology and function. Indeed, in the livers of NASH patients, enlarged Kupffer cells occur in aggregates around the perivenous regions, while in simple steatosis their distribution is more diffuse [71]. There is also evidence that Th1 immune response dominates in NAFLD, promoting the classical M1 activation of Kupffer cells [72, 73]. In addition, recent studies in rodents suggest a direct role for Kupffer cell M1 activation in hepatic fatty acid metabolism and steatosis [74, 75]. Interestingly, Kupffer cells from rats, fed a high-fat diet or challenged with endotoxin, produce high levels of NO and the TNFα M1 cytokine [76, 77]. Moreover, the depletion of Kupffer cells prevents steatosis and the development of insulin resistance [78]. In mice, Kupffer cell depletion is also associated with a decrease in hepatic triglyceride levels and increased expression of key genes involved in fatty acid oxidation, such as PPARα [79]. The ability of PPARs and LXRs to reduce cytokine production in activated inflammatory monocyte-macrophage cells is well documented [45–47]. However, the effects of nuclear receptor agonists on activated Kupffer cells remain unclear. Some studies show that pioglitazone, a clinically available ligand of PPARγ, prevents endotoxin-induced liver injury via a mechanism dependent on suppression of TNFα and NO production by Kupffer cells [76, 77]. In mouse liver, PPARα activation is associated with Kupffer-cell mediated reactive oxygen species production and carcinogenesis [80]. Moreover, case reports indicate that therapeutic use of PPARα ligands can lead to hepatic fibrosis [81]. Contrary to these observations, PPARα upregulation has been shown to ameliorate experimentally-induced liver steatosis in rats [82]. Thus the common thread linking PPARα activation, Kupffer cells, and hepatic pathologies remains undefined [83].

Kupffer cells can also be alternatively activated, and PPARδ has recently been shown to be required for this M2 activation of Kupffer cells (Figure 2(a)). Expression of M2 related genes in Kupffer cells is reduced in PPARδ-deficient lean mice, and transplantation of PPARδ-null bone marrow into wild-type mice is enough to trigger hepatic dysfunction and systemic IR [35], probably due to reduced M2 activation of resident hepatic macrophages. In a similar way, myeloid-specific PPAR$\delta^{-/-}$ mice fed a high-fat diet gain more weight, acquire a higher body weight/liver weight ratio, and have a more profound steatosis than control animals [84]. Moreover, M2 markers are downregulated in these animals. PPARδ is thus an interesting potential pharmacological target for the induction of M2 activation to control inflammation and improve steatosis in NAFLD.

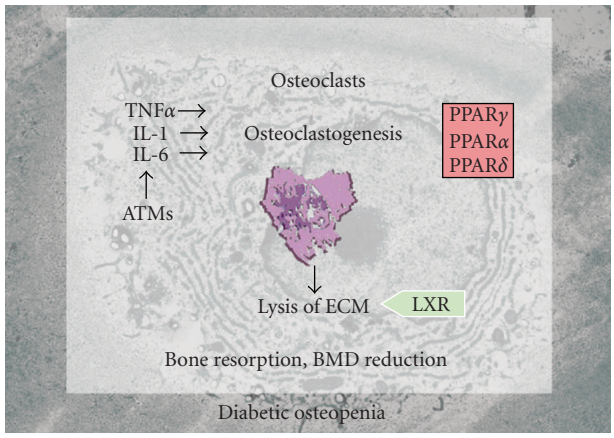
5. Osteoclastogenesis in Obesity Leads to Bone Mass Reduction

Clinical studies indicate that IR conditions such T2DM and severe obesity are associated with increased fracture risk although not always with low bone mass [2, 85–87]. Despite this association, T2DM has been classically coupled to higher bone mineral density (BMD) [88]. It is likely that in humans diabetic bone is more fragile due to changes in bone architecture rather than as a consequence of the reduced BMD. Furthermore, leptin-deficient obese (ob/ob) mice, a model of obesity and IR, have a complex bone phenotype, displaying increased trabecular bone volume in the spine but short femora with reduced cortical thickness and reduced trabecular volume [89]. Therefore, although IR is clearly associated with bone fragility, a direct effect of IR on BMD is highly controversial. There is also a disputed association of obesity-associated bone fragility with several IR-derived defects, such as high insulin levels, low insulin-like growth factor-1 synthesis, low serum adiponectin, and elevated levels of inflammatory cytokines.

Under IR conditions, a compensatory hyperinsulinemia develops. Insulin appears to be anabolic for bone, and recent clinical studies demonstrate that elevated insulin levels can increase BMD [90]. Adiponectin serum levels decrease with obesity, but osteoblasts and osteoclasts express receptors for adiponectin [91, 92], indicating a direct role of this factor in the regulation of bone homeostasis. Some reports linking obesity with increased BMD have demonstrated that adiponectin can promote bone resorption [93, 94]. In contrast, a recent study reports that adiponectin inhibits osteoclastogenesis in primary human cells in vitro and stimulates osteoblast growth [95]. These contradictory results suggest that the direct action of adiponectin on bone increases BMD, but that the final sum of its direct and indirect actions leads to bone mass reduction. Moreover, bone architecture and mechanical properties unrelated to BMD can be impaired in patients with T2DM, possibly due to the lowered levels of insulin-like growth factor-1, a characteristic alteration



(a)



(b)

Figure 2: Tissue-resident macrophages are sources and targets of inflammatory mediators in obesity. (a) Liver-resident macrophages (Kupffer cells) are major sources of inflammatory cytokines in obesity and IR. Free fatty acids (FFAs) and oxidized low density lipoproteins (oxLDL) released from VAT promote M1 phenotype polarization through activation of TLRs. The switch to the M2 phenotype is promoted by PPARδ signaling. Inflammatory mediators (IL-6, TNFα, and IL-1β) originating from M1 Kupffer cells or adipose tissue macrophages (ATMs) induce hepatocyte apoptosis, IR, and lipid accumulation. (b) Osteoclastogenesis is induced by ATM-derived inflammatory cytokines in obesity and IR. Activation of PPARs blocks osteoclastogenesis and impedes bone loss, while LXR promotes osteoclast resorptive activity.

in systemic IR. Finally, inflammatory cytokines, including CRP, IL-1, IL-6, and TNFα, accelerate bone turnover and osteoclastogenesis, and may lead to reduced BMD in humans [96–99]. Indeed, cytokine plasma levels can predict bone resorption in aged adults [100]. These studies illustrate the complexity of bone physiology and its paracrine/endocrine metabolic control, which makes it difficult to clarify the relationship between low bone mass, obesity, and IR.

Nevertheless, increased osteoclast activity and decreased osteoblast differentiation are the basis of BMD loss. Bone homeostasis is maintained by the equilibrium between the activities of bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoclasts are derived from haematopoietic myeloid bone marrow progenitors whereas osteoblasts and adipocytes originate from bone marrow mesenchymal stem cells [101, 102]. Given the importance of crosstalk between macrophages and adipocytes in obesity progression, the fact that macrophages and osteoclasts, and likewise adipocytes and osteoblasts, share common precursors suggests the existence of important interactions between bone and fat. Osteoclasts and osteoblasts also produce factors capable of influencing AT biology, such as osteocalcin or osteopontin. Osteocalcin is secreted by osteoblasts and modulates the expression of various genes in adipocytes and insulin secreting β-cells in pancreatic islets [103]. Osteopontin, which is produced by various cell types such as macrophages, hepatocytes and osteoclasts, promotes inflammation and macrophage accumulation in AT [104]. These findings suggest that bone has endocrine functions through which it might be involved in obesity progression [105]. However, there is little published research into the possible contribution of crosstalk between fat and bone to the regulation of energy balance.

Paradoxically, administration of the insulin sensitizing synthetic ligands of PPARγ can induce bone loss and increase the risk of bone fractures [106, 107]. In mice, activation of PPARγ with TZDs promotes osteoclast differentiation and consequent bone resorption [107]. Consistently, macrophage-specific deletion of PPARγ leads to elevated BMD due to altered osteoclast activity [108]. It is also documented that PPARγ can control osteoblast differentiation from common bone marrow mesenchymal precursors of the osteoblast/adipocyte lineages [109]. Mice with PPARγ haploinsufficiency therefore also have high BMD, coupled with reduced bone marrow adiposity [110]. LXRs are also required for a correct osteoclast function (Figure 2(b)). LXR KO mice show a significantly increased BMD coupled to paradoxically elevated number of osteoclasts in cortical bone, suggesting that LXRs promote osteoclast resorption activity but is not necessary for osteoclast differentiation [111]. Conversely, ligands of PPARγ, PPARα, and PPARδ were recently shown to inhibit the formation of multinucleated osteoclasts from human blood monocytes in vitro [112] (Figure 2(b)).

The use of insulin sensitizing and hypolipidemic drugs, such as PPAR and LXR ligands, might, by decreasing BMD, be related to the increased fracture risk observed in T2DM patients. However, it is still unknown to what extent BMD contributes to increased fracture risk and whether IR has a direct effect on BMD.

6. Concluding Remarks

Insulin resistance is the fundamental cause of a broad range of metabolic abnormalities including glucose intolerance, overt hyperglycemia, hyperinsulinemia, atherogenic dyslipidemia, cardiovascular diseases, kidney disease, liver steatosis,

and osteoporosis. Obesity-associated chronic inflammation is a key contributor to decreased insulin signaling throughout the disease progression, although the specific mechanisms that link inflammation to IR remain not fully understood.

The latest advances in the understanding of macrophage biology place macrophages as the drivers of this inflammatory response. Recruitment of M1 inflammatory macrophages and increased cytokine production in AT and liver not only perpetuate inflammation in these organs but also influence other tissue functions. For instance, obesity-associated inflammatory effects on bone physiology are well documented in many clinical studies. However, the results are controversial and difficult to interpret, and there is therefore a need for further studies to address this question and clarify whether IR has direct effects on bone homeostasis.

The functions of PPAR and LXR nuclear receptors in macrophages include the control of both metabolic and inflammatory pathways. Activation of these receptors thus acts as a link between these two processes closely related to the development of IR. A large body of evidence clearly shows that the insulin-sensitizing properties of NRs are, at least in part, a result of inflammatory control in macrophages. A better understanding of the molecular mechanisms by which NRs control macrophage activation would therefore facilitate the development of pharmacological strategies to specifically target pathways regulating obesity before the onset of obesity-associated complications.

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Review Article

Mediators of Inflammation in Polycystic Ovary Syndrome in Relation to Adiposity

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Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age group and is associated with a higher cardiovascular risk. Obesity, mainly visceral adiposity, is prevalent in patients with PCOS. Obesity is associated with low-grade inflammation and raised inflammatory cytokines, both of which are also described in patients with PCOS. In this paper, the potential relationships between fat distribution, adipocyte dysfunction and, altered inflammatory markers in patients with PCOS have been discussed.

1. Obesity in Polycystic Ovary Syndrome (PCOS)

PCOS is one of the most common endocrine disorders in women of reproductive age with a prevalence of 5%–7% [1–3]. PCOS is associated with a broad range of adverse sequelae, including hypertension, dyslipidemia, insulin resistance, hyperandrogenaemia, gestational and type 2 diabetes, which ultimately increase the risk of cardiovascular morbidity [4–12]. Obesity is present in varying degrees in women with PCOS and is associated with hyperandrogenaemia and insulin resistance [13].

2. Inflammatory Mediators in Obesity

Obesity is associated with a state of chronic systemic inflammation manifested by increased serum levels of inflammatory cytokines as well as alterations in peripheral blood lymphocyte frequencies and function [14–16]. These changes are present not only at the tissue level but also in adipose, liver, and other tissue beds [17, 18]. This inflammatory process could be the underlying cause of obesity-related comorbidities, including atherosclerosis, diabetes and steatohepatosis [19–23].

Obesity-related inflammation is often considered a disorder of innate immunity. However, there is a significant crosstalk between innate and adaptive immune systems, and indeed disorders of both innate and adaptive immunity have been implicated in obesity-related inflammation [17, 24, 25].

Inflammation is not only an acute response to trauma or infection, it is also a response to the ongoing processes of cell turnover associated with aging [26]. In this regard, the inflammatory response regulates fundamental processes intrinsic to cellular homeostasis, including proliferation, necrosis, and apoptosis. In keeping with the task of regulating tissue turnover, inflammatory responses are triggered not only by exogenous stimuli, but also by endogenous stimuli, such as the by-products of cell necrosis and apoptosis. For example, free fatty acids, systemic levels of which are elevated in obesity, are primary ligands for Toll-like receptors, central regulators of the innate immune response [27, 28]. Free fatty acids and Toll-like receptors therefore act as a direct link between the systems that regulate obesity and inflammation.

At the molecular level, the intracellular signalling pathways that govern inflammation and glucose homeostasis demonstrate significant crosstalk and share multiple signalling mediators. At the cellular level, adipocytes and macrophages are closely related and likely evolved from a

common primordial precursor cell [29], further evidence of the parallel evolution of inflammation and metabolic systems.

3. Inflammation and Insulin Resistance in Polycystic Ovary Syndrome

Approximately 60%–70% of PCOS patients are obese [30], with a central body fat distribution pattern described as visceral obesity that is well known to be highly associated with insulin resistance. However, PCOS patients have evidence of insulin resistance independent of obesity [31–33]. Insulin sensitivity is decreased by 35%–40% in women with PCOS, independent of obesity, a decrease similar in magnitude to that seen in T2DM mellitus [34]; however, any degree of obesity further impairs insulin action. About 50%–70% of all women with PCOS have some degree of insulin resistance [35]. It is now evident that PCOS has major metabolic consequences related to insulin resistance. Insulin resistance is associated with an increased risk for several disorders, including type 2 diabetes, hypertension, dyslipidemia (low high-density lipoprotein cholesterol and high triglycerides), elevated plasminogen activator inhibitor type 1 (PAI-1), elevated endothelin-1, endothelial dysfunction, and heart disease.

Data have demonstrated a correlative as well as causative relationship between insulin resistance and inflammation [36]. Subclinical inflammation and insulin resistance are important predictors of cardiovascular disease [37]. Furthermore, in light of the role of insulin resistance in PCOS and of the increased cardiovascular risk of affected women, a relationship between inflammation and hormonal-metabolic features of women with PCOS has been demonstrated [38].

According to Rotterdam consensus criteria commonly used in clinical practice, two of the following three must be fulfilled for the diagnosis of PCOS: polycystic ovaries (12 or more follicles in each ovary, each follicle measuring 2–9 mm in diameter and/or ovarian volume >10 mL, one polycystic ovary is sufficient for the diagnosis), oligo-/anovulation; clinically diagnosed as oligo-/amenorrhea (menstrual cycles longer than 35 days or less than 10 menstruations per year) and hyperandrogenism (clinical or biochemical) [39]. In this consensus insulin resistance, metabolic syndrome, and obesity are not included in the diagnostic criteria to identify PCOS. However it is possible that some phenotypes of PCOS (i.e., those characterised by polycystic ovaries and oligomenorrhea as per Rotterdam consensus criteria) may simply reflect abnormal androgen and/or LH production without having metabolic implications.

It has been reported that women with PCOS have significantly increased hs-C-reactive protein (hs-CRP) concentrations [40], suggesting CRP a marker of low-grade inflammation, as a predictor of coronary heart disease and cardiovascular events in PCOS that is also independently related to insulin resistance. The leukocyte count was found to be significantly higher in women with PCOS compared with healthy women, although no case of leukocytosis was found in either group [38]. Regarding the leukocyte differential, significant increases in lymphocytes and monocytes were observed in women with PCOS compared with controls, which might have been expected considering that they play a key role in the pathophysiological mechanism of atherosclerosis [38]. Inflammation has been recognised to play a central role in both initiation and progression of the atherosclerotic process; therefore, an elevated leukocyte count should be directly associated with increased incidence of coronary heart disease, ischemic stroke, and mortality from cardiovascular disease [41].

In patients with PCOS circulating levels of tumour necrosis factor α (TNF α), interleukin (IL)-6, hs-CRP, as well as white blood count (WBC) and neutrophil count have been found to be elevated compared with age- and /body mass index- (BMI-) matched controls [40, 42, 43]. In contrast, it has been shown that obesity, and not PCOS status per se, was a major determinants of the circulating inflammatory markers TNF α, soluble type 2 TNF receptor, IL-6, and hs-CRP [44, 45]. Increase in both low-grade chronic inflammation and insulin resistance in women with PCOS is associated with increased central fat excess rather than PCOS status [46]. Furthermore, TNF α is over expressed in adipose tissue [47] and induces insulin resistance through acute and chronic effects on insulin-sensitive tissues. The source of excess circulating TNF α in PCOS is likely to be adipose tissue in the obese but remains unknown in lean women with the disorder. However, increased visceral obesity could be a source of excess TNF α in lean women with PCOS.

Another proinflammatory cytokine is IL-18, which was reported to be increased in PCOS [48]. IL-18 induces the production of TNF α which promotes the synthesis of IL-6, which is also considered a strong risk marker for cardiovascular disease [49]. Collectively, the above findings indicate that low-grade chronic inflammation could be a novel mechanism contributing to increased risk of coronary heart disease in PCOS.

Abdominal obesity is largely prevalent in obese women with PCOS [50]. Because of this, it is not surprising that the same alterations of abdominal obesity have been found in obese women with PCOS. In fact, compared with normal weight controls, obese women with PCOS present lower levels of adiponectin [51], increased levels of PAI-1 [52], increased activity of the angiotensin-renin system [53, 54], and increased cytokines and inflammatory markers [41]. However, obese patients with PCOS have more severe insulin resistance and higher androgen levels in comparison with non-PCOS women with abdominal obesity. Since both of these factors may affect adipocyte function, it is important to understand whether there are differences in production of adipose factors between obese women with PCOS and non-PCOS women with abdominal obesity. There were no differences in levels of leptin, resistin, and adiponectin between obese women with PCOS and obese controls [55]. There were also no differences in levels of TNF α, IL-6, and markers of inflammation between obese women with PCOS and obese controls [41, 44]. A significant increase in PAI-1 levels between obese women with PCOS and obese controls has been reported [56]. However, comparing normal-weight patients with PCOS with controls of similar BMI, normoweight women with

PCOS have higher serum levels of PAI-1, TNF α and lower adiponectin than normoweight controls [41, 53, 55]. All these data suggest that normoweight women with PCOS have an increased production of adipokines that is similar to that found in abdominal obesity. Since these patients present a mild hyperinsulinemia and insulin resistance [57], it is possible that it is sufficient to alter the adipocyte function. Consistent with this hypothesis, serum PAI-1 correlates with serum insulin in normoweight women with PCOS [58, 59]. However, in the same group of patients, no correlation was found between serum adiponectin [55] or serum TNF α [41] and serum insulin levels or indices of insulin resistance. Even in overweight patients with PCOS no correlation between serum adiponectin and serum insulin or indices of insulin resistance was found [55].

Abundant leptin receptors have been detected in ovarian granulosa and theca cells [60]; furthermore, leptin treatment of these cells in vitro caused significant reduction in their steroid output [61]. It is possible that leptin has a dual effect on reproduction and that the major site of action differs according to the circulating levels [62]. Initial reports suggested that a substantial proportion of women with PCOS have leptin levels that are higher than expected for their BMI [63]. However subsequent studies have provided evidence that circulating leptin levels are fully accounted for by the degree of adiposity and BMI compared to matching control subjects [64–67]. On the other hand, it has also been reported that, for any given body weight, circulating leptin concentrations are lower in women with PCOS than those without, suggesting that neuroendocrine recognition of obesity may be impaired in such women [68].

Hyperinsulinemia alone is likely not sufficient to explain adipocyte dysfunction of normoweight women with PCOS. Theoretically, normoweight and overweight women with PCOS may have some degree of visceral obesity that is insufficient to effect an increase in body weight per se, but that may be sufficient to determine increased production of some adipokines [55]. On the other hand, visceral obesity and hyperinsulinemia are generally strictly related, and it is difficult to separate the two phenomena.

It has been shown that normoweight patients with PCOS have higher fat accumulation in visceral deposit [69] and lower subcutaneous fat in gluteofemoral area [70]. Although greater experiences and studies on the correlation between fat distribution and adipose products in normo-weight and overweight women with PCOS are needed, the available data suggest that in these patients, increased abdominal fat participates in the increased cardiovascular risk. Of course, insulin resistance is linked to the increase of visceral fat, and it may contribute to the adipocyte dysfunction of normoweight women with PCOS.

In conclusion, patients with PCOS present excessive fat accumulation in visceral deposits, and it plays an important role in their increased cardiovascular disease. This altered fat distribution is present not only in the obese, but also in normoweight patients with PCOS. Altered fat distribution and adipocyte dysfunction along with chronic lowgrade inflammation could be a novel mechanism contributing to increase in cardiovascular risk in PCOS.

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Review Article

Endothelial Dysfunction, Inflammation, and Apoptosis in Diabetes Mellitus

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Endothelial dysfunction is regarded as an important factor in the pathogenesis of vascular disease in obesity-related type 2 diabetes. The imbalance in repair and injury (hyperglycemia, hypertension, dyslipidemia) results in microvascular changes, including apoptosis of microvascular cells, ultimately leading to diabetes related complications. This review summarizes the mechanisms by which the interplay between endothelial dysfunction, inflammation, and apoptosis may cause (micro)vascular damage in patients with diabetes mellitus.

1. Introduction

The rapidly increasing prevalence of diabetes mellitus worldwide is one of the most serious and challenging health problems in the 21st century.

The number of people with diabetes grows faster than expected. In 2007, 246 million people (roughly 6%) were affected worldwide and it is estimated that this will increase to 380 million, or 7.3% by 2025. Furthermore, it is estimated that there are even more people (308 million or 8.1%) with impaired glucose tolerance (IGT). These people have a significant risk of developing type 2 diabetes mellitus (T2DM).

Diabetes is a metabolic disorder which is characterized by hyperglycemia and glucose intolerance due to insulin deficiency, impaired effectiveness of insulin action or, both.

Type 1 diabetes mellitus (T1DM) is caused by cellular-mediated autoimmune destruction of pancreatic islet beta-cells leading to loss of insulin production. It usually starts during childhood, but can occur at all ages. T2DM accounts for 90%–95% of all diabetes and is more common in people older than 45 who are overweight. There is strong evidence that genetics plays an important role as well. However, the prevalence of T2DM is becoming higher in children and young adults because of the higher rate of obesity in this population.

Central obesity and insulin resistance next to diabetes, high cholesterol and high blood pressure form the most important risk factors for cardiovascular disease (CVD). CVD is the major cause of death in people with T2DM. Diabetes is also the leading cause of blindness, renal failure, and lower limb amputations [1, 2].

Dysfunction of the endothelium is regarded as an important factor in the pathogenesis of vascular disease in diabetes mellitus [3–5]. The endothelium is the active inner monolayer of the blood vessels, forming an interface or barrier between circulating blood in the lumen and the rest of the vessel wall, and plays a critical role in vascular homeostasis. It actively regulates vascular tone and permeability, the balance between coagulation and fibrinolysis, the inflammatory activity and cell proliferation. The endothelium even affects the functions of other cell types, such as vascular smooth muscle cells (VSMC's), platelets, leukocytes, retinal pericytes, renal mesangial cells,

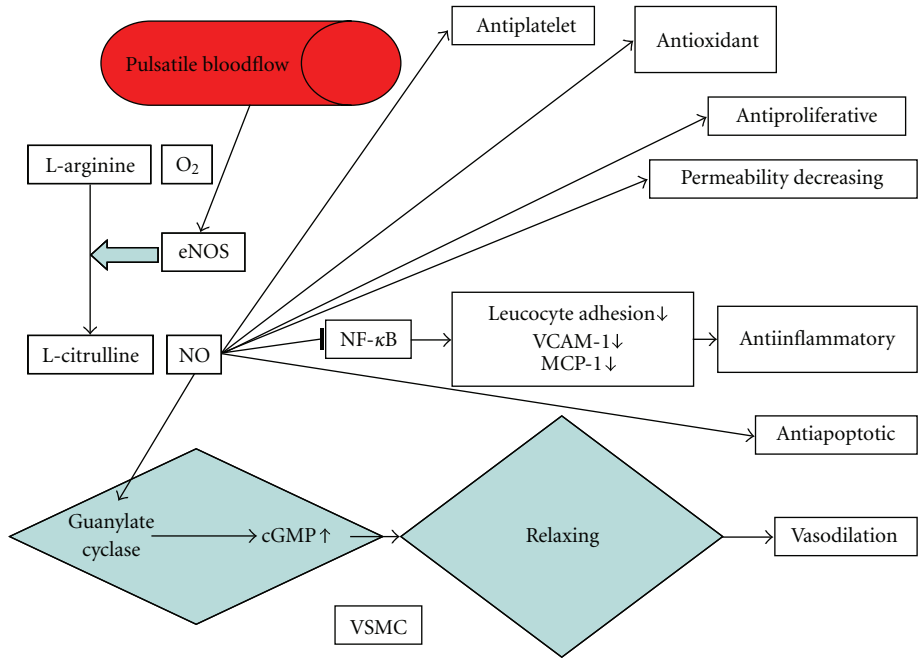

Figure 1: Properties and production process of NO (nitric oxide) as important factor in endothelial function.

and macrophages, amongst others through the production of several chemical mediators [3–8]. In health, endothelial cell injury is mitigated by endogenous reparative processes.

An imbalance in repair and injury resulting in early microvascular changes, including apoptosis of microvascular cells, can be seen in both experimental diabetic animal models and humans with diabetes. Several studies indicate that microvascular cell apoptosis plays an important role in the development of early lesions [6, 8, 9].

We will review the role of endothelial dysfunction and especially inflammation-induced apoptosis of endothelial cells in obesity-related diabetes mellitus and its comorbidities.

2. Endothelial Function and Dysfunction

To maintain vascular homeostasis, the endothelium produces components of the extracellular matrix such as collagen and a variety of regulatory chemical mediators, including nitric oxide (NO), prostanoids (prostacycline), endothelin-1 (ET-1), angiotensin II (ANG-II), tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (vWF), adhesion molecules (VCAM, LAM, ICAM), and cytokines, among them Tumor Necrosis Factor α (TNFα) [10] (Figure 1).

The endothelium has a prominent role in maintaining blood fluidity and restoration of vessel wall integrity to avoid bleeding. It regulates fibrinolysis by producing t-PA and its inhibitor PAI-1 and limits activation of the coagulation cascade by thrombomodulin/protein C, heparin sulphate/antithrombin and tissue factor/tissue factor inhibitor interactions. Through release of promoters and inhibitors of growth and differentiation of the VSMC, such as platelet-derived growth factor (PDGF) and ANG-II, endothelium also has an impact on vascular remodeling [11].

ANG-II exerts regulatory effects on several VSMC activities including contraction, growth, proliferation, and differentiation. By the production of adhesion molecules like leukocyte adhesion molecule (LAM), intracellular adhesion molecule (ICAM), and vascular cell adhesion molecule (VCAM), inflammatory cells are attracted and anchored, thereby playing a regulatory inflammatory role [12, 13].

Endothelial dysfunction is the change of these properties, either in the basal state or after stimulation, that is inappropriate with regard to the preservation of organ function. The kind of changes that occur, can depend on the type of injury and may depend on the intrinsic properties of the endothelium (venous versus arterial endothelium).

Under physiological circumstances, there is a balanced release of endothelial-derived relaxing factors such as nitric oxide (NO) and prostacyclin (PGI2), and contracting factors such as endothelin-1 (ET-1), prostaglandins, and angiotensin II (ANG-II). In endothelial dysfunction, this balance is altered, predisposing the onset and progression of atherosclerosis [14]. Risk factors such as hypercholesterolemia, dyslipidemia, smoking, and diabetes initiate atherosclerosis through endothelial activation and therefore through endothelial dysfunction. Endothelial dysfunction is expressed in increased interactions with leukocytes, smooth muscle growth, vasoconstriction, impaired coagulation, vascular inflammation, thrombosis, and atherosclerosis [15].

A very important mediator synthesized by endothelial cells is nitric oxide (NO), because of its vasodilatory, antiplatelet, antiproliferative, permeability-decreasing, anti-inflammatory, and antioxidant properties [16]. NO inhibits rolling and adhesion of leucocytes as well as cytokine-induced expression of vascular cell adhesion molecule-1

(VCAM-1) and monocyte chemotactic protein-1 (MCP-1) [17], probably through the inhibition of the transcription factor nuclear factor κ B (NF-κB) [14, 18, 19].

NO is produced through the conversion of the amino acid l-arginine to l-citrulline by the enzyme NO-synthase (NOS). There are several isoforms: NOS1 isolated from the brain, NOS2, or iNOS, produced by macrophages and NOS3 or eNOS from endothelial cells. eNOS is activated by the pulsatile flow of blood through vessels. eNOS produces NO which diffuses to the vascular smooth muscle (VSM) where it activates the enzyme guanylate cyclase which in turn increases cyclic GMP and thereby induces relaxation of the VSM. In this way it maintains the diameter of the blood vessel ensuring optimal perfusion of tissues. NOS is regulated by bradykinin, which acts with b2 receptors on the endothelial cell surface membrane, increasing the production of NO via NOS activation. The local concentrations of bradykinin are regulated by the activity of angiotensin converting enzyme (ACE), by breaking down bradykinin into inactive peptides [20, 21].

Endothelial dysfunction is associated with decreased NO availability, either through loss of NO production or through loss of NO biological activity [22]. NO production is diminished in cells which are subject to oxidative stress. Oxidative stress is caused by three factors: (1) an increase in oxidant generation, (2) a decrease in antioxidant protection, (3) a failure to repair oxidative damage. Cell damage is induced by reactive oxygen species (ROS), which are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. Examples are hydroxyl radical, super oxide, hydrogen peroxide, and peroxynitrite. Normally these ROS are scavenged by different intra- and extra cellular mechanisms, but in a situation of oxidative stress these mechanisms are insufficient to cope with the exaggerated generation of ROS. NO may react with some ROS species to form peroxynitrite, in turn increasing the oxidative stress in the cell. Several cardiovascular risk factors like hyperglycemia, insulin resistance, dyslipidemia, inflammation, and also cigarette smoking may induce oxidative stress [5, 19].

Oxidative stress is an important factor which can induce cell apoptosis. In the next part, we will explain the process of apoptosis.

3. Apoptosis

Apoptosis is the process in which a cell plays an active role in its own death. This is why it is also called cell suicide. Apoptosis differs from necrosis in the level of control of the process. Apoptosis is a controlled and regulated process and involves individual cells. Necrosis is an uncontrolled process of cell lysis leading to inflammation and destruction of tissue areas or even whole organs, which can cause serious health problems. Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms and continues throughout adult life. Apoptosis and proliferation are responsible for shaping tissues and organs in developing embryos. During adult life, apoptosis is a protection mechanism which eliminates old, useless, and damaged cells. In healthy organisms apoptosis and cell proliferation are in balance. In diseases such as cancer there is an imbalance whereby cells have undergone certain mutations that prevent them from undergoing apoptosis. In neurodegenerative diseases such as Parkinson's disease apoptosis is thought to account for the excessive loss of neurons.

There are several mechanisms through which apoptosis can be induced in cells. There are extrinsic signals such as the binding of death inducing ligands to cell surface receptors also called death receptors. Some of these ligands are expressed on the surface of cytotoxic T lymphocytes, for example, when a cell is infected by a virus. Apoptosis can also be induced by intrinsic signals, that are produced following cellular stress. Cellular stress can be caused by oxidative stress through free radicals, deprivation of growth factor, or exposure to radiation or chemicals. The sensitivity of cells to these stimuli can vary depending on a number of factors, such as the expression of pro- and antiapoptotic proteins, the severity of the stimulus and the stage of the cell cycle.

Very important death inducing ligands are the Fas ligand, TNFα and TRAIL (TNF related apoptosis inducing ligand). When they bind their specific death receptor, apoptotic signals are transmitted in the cell and a caspase cascade is activated within seconds of ligand binding, inducing apoptosis in a very rapid way. The general signaling pathway that is activated through death receptor binding begins with the generation of ceramide, produced by acid sphingomyelinase. Ceramide release promotes lipid raft fusion which results in clustering of death receptors. This is important because it helps amplify the apoptotic signaling. A conformational change in the intracellular domains of the death receptors reveals the presence of a death domain which allows the recruitment of various apoptotic proteins to the receptor. This is called the death inducing signaling complex (DISC). As a final step, the DISC recruits and activates procaspase 8. Caspase 8 initiates the apoptosis of the cell.

The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and antiapoptotic bcl-2 proteins. Bcl-2 and bcl-XL are antiapoptotic, while Bad, Bax and Bid are proapoptotic proteins [23, 24]. The proapoptotic bcl-2 proteins are often found in the cytosol acting as sensors of cellular damage or stress. In case of cell stress they relocate to the surface of mitochondria where the antiapoptotic proteins are located. This interaction between pro- and antiapoptotic proteins leads to the formation of Permeability Transition pores (PTP) in the mitochondrial membranes [25]. Recent evidence implies that there may also be a mitochondrial apoptotic pathway distinct from that activated by proaptotic bcl-2 family proteins, dependent on cyclophilin D [26]. The mitochondria contains proapoptotic proteins such as Apoptosis Inducing Factor (AIF), Smac/DIABLO, and cytochrome C, which are released through these pores, which in turn leads to the formation of the apoptosome and the activation of the caspase cascade [27, 28].

Once cytochrome C is released into the cytosol, it interacts with apoptotic peptidase activating factor-1 (APAF-1) and this leads to the recruitment of procaspase 9 into a multiprotein complex called the apoptosome. Activation

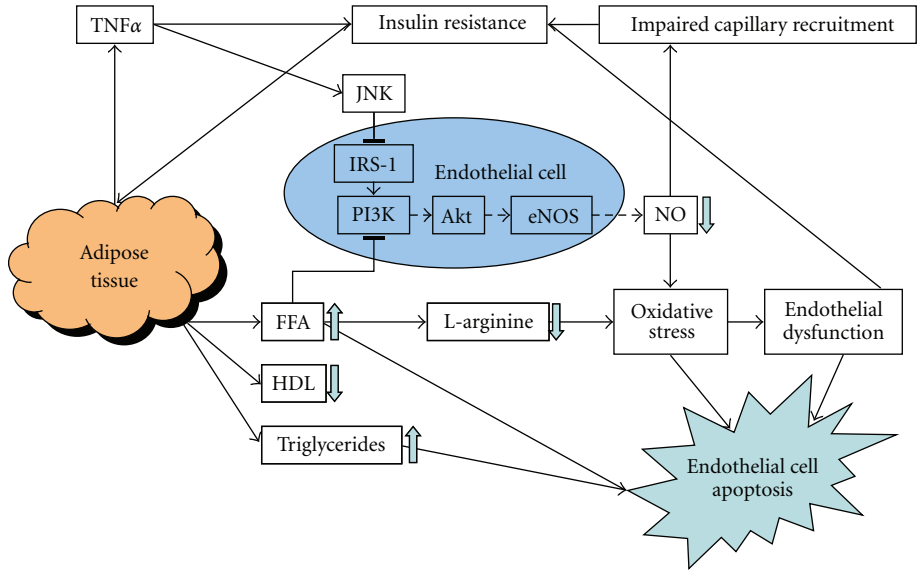

Figure 2: Mechanisms of insulin resistance and adipose tissue in relation to endothelial dysfunction and apoptosis.

of caspase 9 through formation of the apoptosome causes apoptosis.

Nitric oxide has been demonstrated to inhibit apoptosis in a number of cell types including endothelial cells. The antiapoptotic effects can be mediated through mechanisms such as nitrosylation and inactivation of caspase 1, 3 and 8. Other mechanisms include activating p53, upregulating heat shock protein 70, and upregulating antiapoptotic proteins Bcl-2 and Bcl-XL. Through activation of cGMP signaling, caspase activity is suppressed, cGMP-dependent protein kinases are activated and possibly the expression of antiapoptotic proteins increases. Apoptosis and especially apoptosis of endothelial cells may be highly significant in the development of diabetes and atherosclerosis [29].

4. Endothelial Cell Dysfunction and Apoptosis in Diabetes

Dysfunction of endothelium in diabetes mellitus is characterized by changes in proliferation, barrier function, adhesion of other circulating cells, and sensitivity to apoptosis. Furthermore, it is suggested that diabetes mellitus modifies angiogenic and synthetic properties of endothelial cells [30–36].

There is a lot of evidence that endothelial dysfunction is closely connected to the development of diabetic retinopathy, nephropathy, and atherosclerosis in both T1DM and T2DM [4, 37]. But, what are the specific mechanisms that cause this close association between diabetes and endothelial dysfunction? Large clinical trials in both T1DM and T2DM have shown that hyperglycemia plays a big part in the pathogenesis of microvascular complications and is a major causal factor in the development of endothelial dysfunction and endothelial cell apoptosis [5, 38, 39]. However, the exact mechanism of hyperglycemia-related tissue damage and clinical complications remains unclear. There is also a significant role for insulin and especially insulin resistance, as increasing evidence implies that the obesity-related progression of insulin resistance to T2DM parallels the progression of endothelial dysfunction to atherosclerosis. Still this relationship has been difficult to prove because insulin resistance is often accompanied by a cluster of other risk factors as mentioned above.

5. Endothelial Dysfunction and Apoptosis in T2DM

The role of endothelial dysfunction in T2DM is very complicated, due to the many independent factors involved, including ageing, obesity, hyperlipidemia, hypertension, low grade inflammation, insulin resistance, and hyperglycemia [40]. All of these factors are associated with the metabolic syndrome, which usually precedes T2DM. The relationship of endothelial dysfunction and all of these factors is not completely understood despite extensive research. Even the question whether endothelial dysfunction is a consequence or the cause of all the changes occurring in the metabolic syndrome and diabetes cannot be answered easily. In the next few paragraphs we will discuss the relation between endothelial dysfunction and the individual factors mentioned above, starting with insulin resistance.

5.1. Insulin Resistance. Insulin resistance is defined as the decreased ability of insulin to promote glucose uptake in skeletal muscle and adipose tissue and the decreased hepatic output of glucose. This may be present years before the development of abnormal plasma glucose levels becomes evident [41, 42] (Figure 2).

Insulin resistance is associated with an increased free fatty acids (FFA) release from adipose tissue, which results in dyslipidemia, including VLDL-hypertriglyceridemia, high plasma FFA, and low HDL-cholesterol concentrations. High

FFA levels and hypertriglyceridemia are associated with endothelial dysfunction. FFA-mediated endothelial dysfunction is probably caused by reduced availability of L-arginine and/or NO and oxidative stress [43]. It has been proven that increased saturated and polyunsaturated FFA concentrations, except for oleic acid, directly induce cell cycle arrest and apoptosis in vascular endothelial cells [44].

Insulin is a vasoactive hormone and enhances muscle blood flow and vasodilation via stimulation of NO production. The increased blood flow caused by insulin, differs among different types of vessels. Insulin can also redirect blood flow in skeletal muscles so that more glucose can be uptaken by muscle cells. This process is called capillary recruitment. In T2DM, hypertension and obesity, insulin's vasodilator actions are impaired, probably for a large part because of low NO action. Normally, stimulation of NO production by insulin is mediated by signaling pathways involving activation of Phosphoinositide-3 (PI-3) kinase leading to phosphorylation of eNOS. It is suggested that endothelial dysfunction and impaired capillary recruitment can cause insulin resistance because the microvascular endothelium can not react properly to insulin and glucose disposal is decreased. This is called endothelial insulin resistance. How metabolic and endothelial insulin resistance originate and their exact relationship are not fully understood. Both TNFα and nonesterified acids (NEFAs) can cause metabolic and endothelial insulin resistance. Inflammatory cytokines like TNFα, can act as mediators of insulin resistance by impairing the tyrosine kinase activity of both the insulin receptor (IR) and insulin receptor substrate (IRS-1), thus inhibiting insulin signaling. It is suggested that a bidirectional relationship exists between hyperinsulinemia and low-grade chronic inflammation, by which hyperinsulinemia can lead to vascular inflammation and vascular inflammation causes insulin resistance and finally compensatory hyperinsulinemia. At normal physiological concentrations insulin exerts prevailing antiinflammatory effects, while hyperinsulinemia increases levels of oxidative stress and inflammation. A recent study with Human Umbilical Vein Endothelial Cells (HUVECs) shows that insulin, at pathophysiological concentrations alone or in combination with low concentrations of TNFα, has the ability to promote VCAM-1 expression, through increasing the steady state levels of mRNA via the activation of transcription factors, such as NF-κB, which has been linked to VCAM-1 transactivation before. This way, hyperinsulinemia leads to increased monocytoid cell adhesion to HUVECs [5, 19, 45].

A very important effect of insulin resistance is the fact that the normal route for insulin to activate the PI-3 kinase and Akt-dependent signaling pathways is impaired, whereas hyperinsulinemia overactivates Mitogen activated protein kinases (MAPK)-pathways, thereby creating an imbalance between PI-3 kinase and MAP-kinase-dependent functions of insulin. This probably leads to decreased NO production and increased ET-1 secretion, characteristic of endothelial dysfunction. Through activation of the MAP-kinase signaling pathways, hyperinsulinemia promotes secretion of ET-1, activates cation pumps, and increases expression of VCAM-1 and E-selectin [46]. ET-1, a vasoconstrictor, can increase serine phosphorylation of IRS-1, causing a decreased activity of PI-3 kinase in vascular smooth muscle cells. Moreover, ET-1 may also impair insulin-stimulated translocation of GLUT-4 in adipocytes [47, 48].

5.2. Hypertension. Hypertension induces endothelial activation and probably also endothelial dysfunction and is a major determinant of microangiopathy and atherothrombosis in diabetes. Hypertension is associated with insulin resistance and this relation can partly be explained by decreased capillary density and impaired capillary recruitment seen in insulin resistant states. Another explanation is the fact that NO availability is diminished and ET-1 availability is increased in both insulin resistance and hypertension. The exact link between diabetes and hypertension is not fully known [49].

5.3. Obesity. The adipose tissue has become known to be a highly active endocrine organ, releasing hormones, cytokines, and enzymes with the tendency to impair insulin sensitivity. It is an important modulator of endothelial function via secretion of a variety of hormones, including adiponectin, resistin, leptin, PAI-1, angiotensin, estradiol, and the cytokines TNFα and interleukin-6 (IL-6). Plasma adiponectin levels are reduced in people with obesity and also in people with diseases associated with obesity, like T2DM and coronary artery disease. Adiponectin has antiinflammatory features and is inversely related to BMI, oxidized LDL, insulin resistance, and atherosclerosis [19]. It plays an important role in fatty acid metabolism and glucose homeostasis. Low adiponectin levels are associated with an increased oxidative state in the arterial wall and systemic oxidative stress. In endothelial cells, adiponectin increases the production of nitric oxide and suppresses oxidative stress and the inflammatory signaling cascades via AMP-activated protein kinases (AMPK) and the cyclic AMP-protein kinase A-linked pathway [50]. Moreover, it reduces the attachment of monocytes to endothelial cells and inhibits the expression of adhesion molecules [5, 51].

The role of resistin in insulin resistance and diabetes is controversial since a number of studies have shown that resistin levels increase with increased central adiposity and other studies have demonstrated a significant decrease in resistin levels in increased adiposity. PAI-1 is present in increased levels in obesity and the metabolic syndrome. It has been linked to the increased occurrence of thrombosis in patients with these conditions.

Angiotensin II is also present in adipose tissue and has an important effect on endothelial function. When angiotensin II binds the angiotensin II type 1 receptor on endothelial cells, it stimulates the production of ROS via NADPH oxidase, increases expression of ICAM-1 and increases ET-1 release from the endothelium [52–54]. Angiotensin also activates JNK and MAPK pathways in endothelial cells, which leads to increased serine phosphorylation of IRS-1, impaired PI-3 kinase activity and finally endothelial dysfunction and probably apoptosis. This is one of the explanations why an ACE inhibitor and angiotensin II type 1 receptor

blockers (ARBs) protect against cardiovascular comorbidity in patients with diabetes and vice versa [55].

Insulin receptor substrate 1 (IRS-1) is a protein downstream of the insulin receptor, which is important for signaling to metabolic effects like glucose uptake in fat cells and NO-production in endothelial cells. IRS-1 in endothelial cells and fat cells can be downregulated by stressors like hyperglycemia and dyslipidemia, causing insulin resistance and endothelial dysfunction. A low adipocyte IRS-1 expression may thereby be a marker for insulin resistance [19, 56, 57].

5.4. Inflammation. Nowadays atherosclerosis is considered to be an inflammatory disease and the fact that atherosclerosis and resulting cardiovascular disease is more prevalent in patients with chronic inflammatory diseases like rheumatoid arthritis, systemic lupus erythematosus and ankylosing spondylitis than in the healthy population supports this statement. Inflammation is regarded as an important independent cardiovascular risk factor and is associated with endothelial dysfunction.

Interestingly, a study performed by bij van Eijk et al. shows that patients with active ankylosing spondylitis, an inflammatory disease, also have impaired microvascular endothelium-dependent vasodilatation and capillary recruitment in skin, which improves after TNFα-blocking therapy with etanercept [58].

The existence of chronic inflammation in diabetes is mainly based on the increased plasma concentrations of C-reactive protein (CRP), fibrinogen, interleukin-6 (IL-6), interleukin-1 (IL-1), and TNFα [59–61]. Inflammatory cytokines increase vascular permeability, change vasoregulatory responses, increase leukocyte adhesion to endothelium, and facilitate thrombus formation by inducing procoagulant activity, inhibiting anticoagulant pathways and impairing fibrinolysis via stimulation of PAI-1. NF-κB consists of a family of transcription factors, which regulate the inflammatory response of vascular cells, by transcription of various cytokines which causes an increased adhesion of monocytes, neutrophils, and macrophages, resulting in cell damage. On the other hand, NF-κB is also a regulator of genes that control cell proliferation and cell survival and protects against apoptosis, amongst others by activating the antioxidant enzyme superoxide dismutase (SOD) [62]. NF-κB is activated by TNFα and IL-1 next to hyperglycemia, AGEs, ANG-II, oxidized lipids, and insulin. Once activated, NF-κB translocates from the cytoplasm to the nucleus to activate gene transcription. NF-κB-regulated genes are VCAM-1, E-selectin, ICAM-1, IL-1, IL-6, IL-8, tissue factor, PAI-1, and NOS.

The TNF-family of cytokines plays an important role in regulating the immune response, inflammation, and apoptosis. The first cytokine discovered is TNFα, which is produced by neutrophils, macrophages, and adipocytes and can induce other powerful cytokines such as IL-6, which in turn regulates the expression of C-reactive protein (CRP). CRP increases the expression of endothelial ICAM-1, VCAM-1, E-selectin, MCP-1 and increases the secretion of ET1. Moreover, CRP decreases eNOS expression and elevates the expression of angiotensin receptor type 1 in the vessel wall [63, 64].

TNFα can induce insulin resistance and this is probably a part of the explanation why insulin resistance, endothelial dysfunction, and atherothrombosis are so closely related. Recent studies indicate that TNFα is likely involved in the pathogenesis of diabetic nephropathy and retinopathy. A very recent study with T1DM and T2DM rats shows that TNFα plays an important role in microvascular apoptosis in diabetes. When the diabetic rats were treated with pegsunercept, a TNFα inhibitor, a significant reduction of the number of endothelial cells that expressed activated caspase-3 by 76% to 80% occurred. TNFα inhibition decreases intercellular adhesion molecule 1 (ICAM-1) levels and NF-κB activity in diabetic retina. Another study in diabetic rats demonstrated that increased levels of TNFα consequently enhanced FOXO-1 mRNA levels, nuclear translocation, and DNA binding in retinas of T1DM and T2DM rats. It also showed that the transcription factor FOXO-1, which regulates cell death; prevents cell cycle progression, modulates differentiation in various cell types, plays a critical role in diabetes-induced apoptosis and retinal microvascular cell loss [65]. It is possible that TNFα upregulation may contribute to increased apoptosis detected in other diabetes associated complications and TNFα inhibition may be a potential therapeutic option in preventing this comorbidity [66].

Tumor necrosis factor alpha-Related Apoptosis-Inducing Ligand (TRAIL), also known as APO2L, is another member of the TNF family of cytokines and is a type II membrane protein. The effects induced by TRAIL are mediated by interactions with cell surface TRAIL receptors. Five TRAIL receptors have been found so far in humans. When TRAIL binds TRAIL-R1 (DR4) and TRAIL-R2 (DR5) apoptotic signals are transduced. TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and osteoprogeterin (OPG) lack an intracellular death domain and can not induce apoptosis. Uniquely, TRAIL can exert anticancer activity, while causing no or minimal organ toxicity and inflammation. TRAIL acts among others on nuclear factor kappa B (NF-κB). TRAIL induces the release of NO by vascular endothelial cells [67]. Studies have shown that OPG is remarkably increased in diabetic patients and even more so in patients with cardiovascular disease, like coronary artery disease or abdominal aortic aneurysm [68, 69]. In a study with SZT-induced rats and a control group of healthy rats the OPG/TRAIL ratio was markedly increased in the diabetic animals with respect to the control animals. The next remarkable observation in this study was the ability of insulin to downregulate TRAIL expression in rat aortas in vivo.

Further investigation of the role of insulin in the TRAIL expression in diabetes was done with VSMCs in vitro. This showed the same result: a decrease of surface TRAIL expression. High glucose levels did not show any significant effect on TRAIL surface expression in both studies. These findings suggest that the downregulation of TRAIL expression may play a role in diabetic vasculopathy. A possible explanation for these results is the upregulation of the transcription factor early growth response protein 1 (Egr-1), which in turn downregulates TRAIL expression in endothelial cells, by

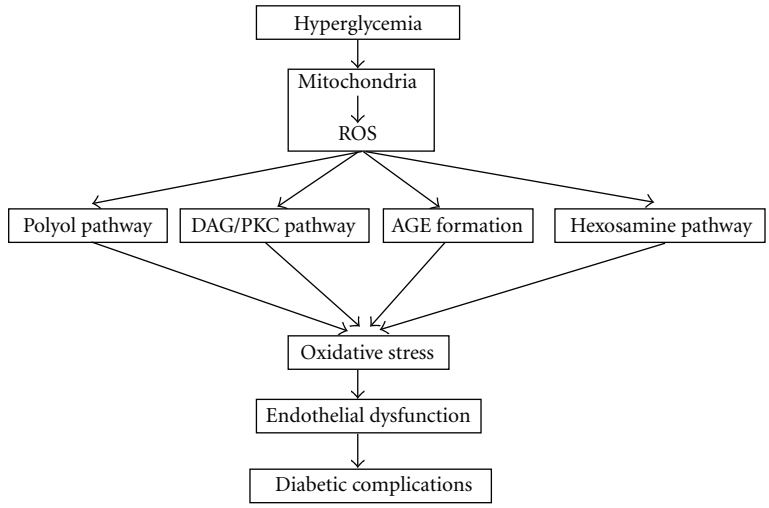

Figure 3: Mechanisms of hyperglycemia which are supposed to cause endothelial dysfunction and in the end diabetic complications.

both hyperglycemia and insulin. A supportive finding for this hypothesis is the fact that VEGF receptor 1 (FLT1) and PAI-1, both known Egr-1 responsive genes, are also increased in the presence of glucose and insulin. Thus, Egr-1 upregulation, which is frequently observed in atherosclerosis, is likely to be involved in insulin-mediated TRAIL downregulation [70].

Plasma levels of C-reactive protein (CRP) are increased in both T1DM and T2DM. CRP plays a significant role in atherogenesis in endothelial cells, next to vascular smooth muscle cells and macrophages, and several studies have revealed that CRP levels predict cardiovascular disease [71]. CRP causes numerous proinflammatory and pro-atherogenic effects in endothelial cells, such as decreased NO and prostacyclin, increased ET-1, cell adhesion molecules, MCP-1, IL-8, and PAI-1 [5].

Another important contribution to chronic inflammation in diabetes is caused by primed peripheral polymorphonuclear leukocytes (PMNs). In a small study with T2DM patients and a control group, it was shown that T2DM patients are exposed to oxidative stress and chronic inflammation partially because of the primed state of their PMNs, amongst others because these primed PMNs release superoxide significantly faster than normal control PMNs. Apoptosis in primed PMNs was also higher in the diabetic patients, probably partly because of intracellular factors such as high cytosolic calcium concentrations [72]. At the same time apoptosis of normal PMNs of the control group was significantly higher in diabetic serum, suggesting leucoclastic activity of diabetic serum. This was confirmed by the findings of Abu El-Asrar et al. [73]. This study also observed a decrease in plasma gluthathione (GSH), an intra- and extra cellular antioxidant, which neutralizes oxidants, including hydrogen peroxide and superoxide, by converting them to other oxidized forms [61].

5.5. Dyslipidemia. Dyslipidemia is characterized by low HDL-cholesterol levels and an excess of small, dense LDL and is associated with obesity, insulin resistance and diabetes in general. An increase in postprandial triacylglycerol-rich lipoproteins, like chilomicrons and -LDL particles, enhances oxidative stress and consequently causes endothelial dysfunction and increased apoptosis [74].

6. Hyperglycemia and Endothelial Dysfunction

There have been various mechanisms discovered that can explain how hyperglycemia causes vascular complications. There are several pathways which get activated through hyperglycemia and can potentiate each other. The basis for the activation of these pathways is most likely the overproduction of ROS in mitochondria induced by hyperglycemia (Figure 3).

6.1. The Polyol/Sorbitol/Aldose Reductase Pathway. In a lot of cells excess glucose is reduced to sorbitol by aldose reductase. Sorbitol is later metabolized to fructose by sorbitol dehydrogenase, the polyol pathway. At the same time it increases the oxidation of NADPH to NADP+ and the reduction of NAD+ to NADH, the co-factors, which in turn decreases NO bioavailability [75]. This causes a redox imbalance that resembles tissue hypoxia and is therefore called hyperglycemic pseudohypoxia. It also increases the formation of methylglyoxal and AGEs. All these processes enhance oxidative stress [76]. The increased sorbitol accumulation increases osmotic stress and decreases other osmolytes such as myo-inositol and taurine. A study in rat and human retinas produced evidence that the polyol pathway may have an important role in diabetic retinopathy. It also proved that the aldose reductase inhibitor (sorbinol) prevents vascular processes, culminating in the development of acellular capillaries [5, 75, 77]. This may imply that the polyol pathway can cause endothelial cell apoptosis. However, the full impact of this pathway in the endothelial dysfunction is not completely understood yet.

6.2. The DAG/PKC Pathway. The hyperglycemia induced activation of the diacylglycerol (DAG)-protein kinase C

(PKC) pathway has multiple adverse effects on the vascular function. Hyperglycemia increases the levels of DAG, which in turn activates PKC. In hyperglycemic circumstances DAG is synthesized from the glycolytic intermediates dihydroxyacetone phosphate (DHAP) and glycerylaldehyde-3-phosphate, by a de novo pathway [78]. Oxidants like H_2O_2 can also activate the DAG/PKC pathway. There are at least eleven PKC isoforms. In vascular cells the isoform PKC-beta-II is most frequently activated [79]. The pathogenic consequences of PKC activation include dysregulation of the vascular permeability through the induction of vascular endothelial growth factor (VEGF) in smooth muscle cells [80], dysregulation of blood flow by decreasing endothelial NOS activity and/or increasing ET-1 synthesis [81], basement membrane thickening through Transforming Growth Factor-beta (TGF-β)-mediated increased production of type IV collagen and fibronectin, increased expression of PAI-1 which causes impaired fibrinolysis and activation of superoxide producing enzymes like NADPH as well as an increased expression of a dysfunctional, superoxide-producing, uncoupled endothelial NOS, thus increasing oxidative stress [5].

Recently, Geraldes et al. have identified a new signaling pathway by which hyperglycemia causes increased vascular cell pathology and apoptosis resulting in diabetic retinopathy in mouse retinas. They proved that hyperglycemia, especially in pericytes, activates PKC-δ, probably through an increase in transcription of the gene encoding PKC-δ. This as well as activation of p38α MAPK leads to increased expression of Scr homology-2 domain-containing phosphatase-1 (SHP-1), which subsequently induces apoptosis via deactivation of platelet-derived growth factor β (PDGF-β) [82].

6.3. Non-Enzymatic Glycation End Products (AGE). Non-enzymatic glycation products are a complex and heterogeneous group of compounds which accumulate in plasma and tissues in diabetes and renal failure. There is emerging evidence that these compounds play a role in the pathogenesis of chronic complications associated with diabetes and renal failure. Earlier research in both diabetic animals and humans revealed an association between the accumulation of AGE-modified proteins and the severity of microvascular complications. The second evidence stems from the fact that typical microvascular complications develop following injections of AGE-modified proteins in non-diabetic animals [83].

The advanced glycation end-products (AGE) concept proposes that chemical modification and cross linking of tissue proteins, lipids, and DNA affect their structure, function and turnover, contributing to a gradual decline in tissue function and to the pathogenesis of diabetic complications. Nonenzymatic glycation of proteins is a condensation reaction between the carbonyl group of free glucose and the N-terminus of reactive-protein amino groups, like lysine or arginine, yielding Schiff-base intermediates that undergo Amadori rearrangement to form stable proteinglucose adducts, for example glycated hemoglobin A1c (HbA1c) and fructosamine (fructoselysine). Amadori-modified matrix proteins are increased in diabetes. Because Amadori-adducts are relatively stable, only a small fraction undergoes rearrangements to irreversible AGEs. At first it was believed that AGEs are only formed on long-lived extra cellular molecules, because of the slow rate of reaction of glucose with proteins. However, other sugars like glucose-6-phosphate and glyceraldehyde-3-phophate can also create AGEs with intracellular and short-lived molecules and at a much faster rate than glucose. AGEs can arise from the decomposition of Amadori products, from fragmentation products of the polyol pathway, and as glycoxidative products which all react with protein amino groups. When oxidation is involved, the so-called glycoxidation products such as pentosidine and carboxymethyllysine are formed. It has recently been found that glucose can probably autoxidize to form reactive carbonyl compounds (glyoxal, methylglyoxal and 3-deoxyglucosone) which may react with protein to form glycoxidation products. In endothelial cells methylglyoxal is probably the main AGE formed. AGEs can interfere with the endothelial function in several ways. They can act as oxidants and cause generation of reactive oxygen species (ROS). AGEs can decrease arterial elasticy and AGE modified type I and IV collagen can prevent normal matrix formation and cross-linking. Interactions of mononuclear cells and macromolecules like LDL with the endothelial wall are stimulated by AGE-modified matrix, through increased expression of endothelial adhesion molecules. AGEs can also impair the binding of heparan sulfate to the extra cellular matrix, which results in a loss of anionic sites and thus in an increase in endothelial permeability. Early diabetic micro angiopathy is characterized by vasodilation, increased blood flow, and increased capillary permeability. AGE-modified proteins may lead to all these changes.

When AGEs get into the blood circulation they are highly reactive but are often detoxified by various enzymes. When they are not eliminated by the kidneys, recirculating AGE peptides can generate new AGEs reacting with plasma or tissue components. At this stage glycation accelerates the progress of deterioration. Age-modified plasma proteins can bind to AGE receptors (RAGE = AGE-receptor, macrophage scavenger receptor A) on different cell types like endothelial cells, where it can adversely affect the expression of thrombomodulin, tissue factor, and VCAM-1 genes. RAGE-binding mediates signal transduction via a receptor-mediated induction of ROS and activation of transcription factors NF-κB and p21-ras, leading to apoptosis [84].

The nonenzymatic glycation of LDL (gLDL) and its role in the pathogenesis of atherosclerosis is a popular subject in studies of late. Due to hyperglycemia, LDL glycation is increased in diabetic patients, however nonenzymatic glycation of LDL happens naturally in all individuals. The modification of LDL by glycation leads to a decreased recognition of LDL by the LDL receptor (LDL-R) and in turn increases the relative circulation time of the lipoprotein, which may result in increased particle oxidation, the formation of AGEs, and the activation of alternative uptake mechanisms by non—LDL-R—mediated pathways. Additionally, gLDL prevents shear stress-mediated L-arginine uptake and nitric oxide formation and causes increased production of plasminogen-activator inhibitor 1 and prostaglandins, while inhibiting

the expression of tissue plasminogen activator in endothelial cells [85–87]. Finally gLDL reduces proliferation and triggers apoptosis in HUVECs [44].

It has been proposed that these processes could contribute to the increased susceptibility of diabetic patients to atherosclerosis and coronary heart disease.

So measurement of the products of nonenzymatic glycation has a two-fold meaning: on one hand, measurement of early glycation products can estimate the extent of exposure to glucose and the subjects of previous metabolic control; on the other hand, measurement of intermediate and late products of the glycation reaction is a precious instrument in verifying the relationship between glycation products and tissue modifications.

6.4. Hyperglycemia and Oxidative Stress. A single unifying mechanism of the above mentioned pathways has recently been found. The increased production of superoxide anion radicals by mitochondrial electron transport chain plays a key role in the activation of the above pathways. Hyperglycemia-induced superoxide overproduction inhibits GADPH activity by 66%, which is a consequence of poly ADP-ribosylation of GADPH by poly ADP-ribose polymerase (PARP), which in turn is activated by DNA strand-breaks synthesized by mitochondrial superoxide overproduction. This overproduction particularly happens in mitochondria that have been uncoupled by the flux of NADH from the hyperglycemia-enhanced glycolysis. GADPH inhibition causes accumulation of glycolysis intermediates. In aortic endothelial cells, the hyperglycemia induced increased mitochondrial superoxide production and prevented eNOS activity and expression [88]. In addition to mitochondrial uncoupling there are other mechanisms that can contribute to superoxide production in diabetes, namely, uncoupling of eNOS, increased peroxidation and glycoxidation, activation of NADPH oxidases, decreased clearance of superoxide, and impaired antioxidant status [61]. Increased production of ROS causes oxidative stress. Oxidative stress is probably a key event in endothelial dysfunction since inhibition of hyperglycemia, induced, ROS production prevents activation of the aldose reductase, hexosamine pathways, PKC activation, and AGE formation [77, 89]. ROS at low concentrations can function as signaling molecules and participate as signaling intermediates in the regulation of fundamental cell activities, such as cell growth and cell adaptation responses. At higher concentrations they can cause oxidative stress, cellular injury, and apoptosis [7, 90]. ROS can effect many signaling pathways, including G-proteins, protein kinases, ion channels and transcription factors. Finally ROS can modify endothelial function by a variety of mechanisms, like peroxidation of membrane lipids, activation of NF-κB, and decreasing the availability of NO [91]. A recently published study showed that transient exposure of cultured human aortic endothelial to hyperglycemia induces persistent epigenetic changes in the promoter of the NF-κB p65 subunit. In the proximal promoter region of p65, increased monomethylation of histone 3 lysine 4 by the histone methyltransferase Set 7 caused a continuing increase in p65 gene expression, leading to a sustained increase in the expression of the NF-κB-responsive proatherogenic genes MCP-1 and VCAM-1. The cause of these changes was found in the increased generation of methylglyoxal and hyperglycemic-induced ROS formation by the mitochondrial electron transport chain. This means that transient hyperglycemia can cause persistent atherogenic effects during normoglycemia by inducing long lasting chromatin remodeling and vascular epigenetic changes. These results provide a molecular basis for better understanding of the variation in risk for diabetic complications, which can not be explained by HbA1c [92].

Oxidative stress is known to induce senescence prematurely in fibroblasts. Cellular senescence or cellular ageing is the phenomenon where normal diploid differentiated cells lose the ability to divide. This phenomenon is also known as "replicative senescence" or the "Hayflick phenomenon". In response to DNA damage (including shortened telomeres) cells either age or go into apoptosis if the damage cannot be repaired. There is strong evidence as mentioned above, that oxidative stress is increased in diabetic patients. Other studies have revealed that endothelial cells in atherosclerotic lesions show features of cellular senescence, like senescence associated β-galactosidase (SA-β-gal) staining and telomere shortening. Expression of inflammatory cytokines and adhesion molecules is upregulated in senescent endothelial cells. Furthermore, nitric oxide production is significantly reduced in these cells. More importantly, senescence enhances vascular inflammation and thrombosis in vessels, promoting the development of cardiovascular events. There is also evidence that senescence is more accelerated in patients with diabetes compared to healthy individuals. One study demonstrated that high glucose induced premature cellular senescence in HUVECs through the activation of the Apoptosis Signal-Regulating Kinase 1 (ASK1). Activation of ASK-1 also upregulated PAI-1 expression in the HUVECs and this plus senescence was also observed in aortas of STZ-diabetic wild type mice, whereas this was not seen in STZ-diabetic ASK-1 knock-out mice. PAI-1 is known to play an important role in the pathogenesis of atherosclerosis and thrombosis [93].

7. Hyperglycemia and Apoptosis

The number of (in vitro) studies delivering evidence that hyperglycemia can induce endothelial cell apoptosis [30, 90, 94] has increased extensively over the last few years. These studies have focused mainly on human or animal endothelial cells of kidney, retina, myocardium, and human umbilical vein endothelial cells (HUVECs). Thanks to these studies, the mechanisms by which hyperglycemia initiates apoptosis are better understood. These mechanisms include oxidative stress, increased intracellular Ca^{2+}, mitochondrial dysfunction otherwise known as the mitochondria apoptosis pathway, changes in intracellular fatty acid metabolism, activation of Mitogen activated protein kinases (MAPK) signaling pathways, and impaired phosphorylation activation of the protein kinase Akt [24, 31] (Figure 4).

One specific study with HUVECs demonstrated that elevated glucose induces apoptosis and downregulates VEGF in HUVECs by inhibiting p42/44 MAP kinase activation. High glucose also significantly increased Bax protein but did not

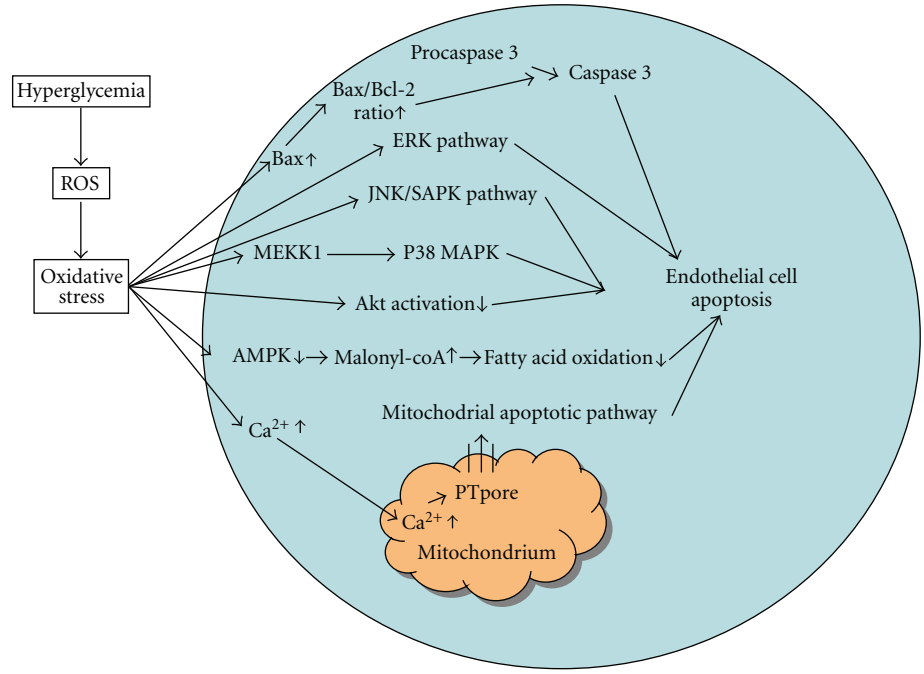

Figure 4: Mechanisms by which hyperglycemia is supposed to induce endothelial cell apoptosis.

affect Bcl-2, thereby elevating the Bax/Bcl-2 ratio which activates cleavage of procaspase 3 into active caspase-3, in turn triggering apoptosis in HUVECs. When VEGF was added to the HUVECs exposed to high glucose, apoptosis was prevented through inhibition of elevated ROS generation, calcium overload and activation of the mitochondria apoptosis pathway. VEGF significantly decreased Bax expression without affecting the Bcl-2 level and attenuated the increase in caspase 3 activity. VEGF in HUVECs could also decrease H_2O_2 production at 48 hours high glucose stimulation, suggesting that it inhibits the ROS/NF-κB/JNK/Caspase-3 pathway [24].

One earlier study with human aortic endothelial cells and bovine aortic endothelial cells exposed to high D-glucose also showed a significant increase in the Bax/Bcl-2 ratio followed by an increase in caspase-3 activity and cell death. They proved that Bax inserts the mitochondrial membranes, triggering a transformation of mitochondrial function after high D-glucose treatment of the human aortic endothelial cells. This study also demonstrated that high D-glucose leads to phosphorylation of p38 Mitogen-Activated Protein Kinase (p38 MAPK) mediated by MEK-kinase1 (MEKK1) downstream of bax-caspase proteases and thereby causes apoptosis of aortic endothelial cells [27].

Another study with HUVECs also investigated the role of the three MAPK pathways: the extra cellular signal-regulated kinases (ERK), the c-Jun NH 2-Terminal Kinase /stress-activated protein kinases (JNK/SAPK), and p38 MAPK. They found that high glucose triggers apoptosis via ROS through activating JNK/SAPK. This study showed no significant role for the other two MAPK pathways [95]. Later in 2005 this research group found that hyperglycemia induces ROS generation through a PI3K-dependent pathway. They observed that hyperglycemia causes a PI3K/Akt-dependent upregulation of Cyclooxygenase 2 (COX-2) expression and thereby an increase of prostaglandin E2 (PGE2) production and subsequently a caspase-3 activation and facilitation of apoptosis in HUVECs. These findings were supported by the fact that LY294002 or wortmann (both PI3K/Akt inhibitors) prevented the COX-2 mediated PGE2 production and subsequently the caspase-3 activity, and apoptosis. Inhibition of COX-2 with a selective COX-2 inhibitor NS398 also inhibited PGE2 production, caspase-3 activity and apoptosis in HUVECs treated with high glucose levels. Moreover they found that hyperglycemia could trigger NF-κB activation and that dominant-negative IkBα could prevent COX-2 expression and apoptosis, implying that NF-κB activation can lead to COX-2 mediated PGE2 production and apoptosis in HUVECs exposed to hyperglycemia [96].

There are several studies with HUVECs that prove that high glucose-induced apoptosis is associated with an increase in Ca^{2+} current, resulting from Ca^{2+} entry mediated by store-operated channels. An increased amount of cellular Ca^{2+} causes more mitochondrial Ca^{2+} uptake. Ca^{2+} accumulation in mitochondria is one of the primary causes for mitochondrial permeability transition, through the opening of the PT-pore and this is an important key factor in the apoptotic pathway [24].

The involvement of the intracellular fatty acid metabolism is suggested by a study in which HUVECs were treated with high glucose concentrations for 24 hours and showed inhibition of fatty acid oxidation, increases in fatty acid esterification and the concentration of malonyl-CoA before apoptosis was induced. This finding suggests a causal relation of alterations in intracellular fatty acid and apoptosis in hyperglycemia. Decreases in mitochondrial membrane

potential and cellular ATP content also preceded apoptosis. All these metabolic alterations are associated with an increase in caspase-3 activity and an impaired ability of insulin at a physiological concentration to activate Akt. Finally an antiapoptotic role for AMPK is suggested in this study because incubation of the HUVECs with 5-aminoimidazole-4-carboxamide-riboside (AICAR), an AMPK activator, prevented all of the above changes. Likewise, a similar decrease in caspase-3 activity was observed when AMPK activity was increased by infecting HUVEC with constitutively active AMPK using an adenoviral vector [33].

Recently, a study with human pancreatic islet microvascular endothelial cells (MECs) proved that sustained hyperglycemia progressively affects cellular survival and proliferation and increases apoptosis of cultured MECs. After 24 to 48 hours, apoptosis was detected in high glucose both by DNA fragmentation and activation of the caspase family. In this study they found that the islet MECs, under conditions of sustained hyperglycemia, showed a progressively reduced phosphorylation of Akt, suggesting an interference with the pathways involved in Akt activation. Hyperglycemia also downregulated the tyrosine phosphorylated form of the transmembrane protein nephrin. It is known that phosphorylated nephrin associates with PI3K and activates the multifunctional Akt-dependent pathways. This suggests that hyperglycemia-induced apoptosis of islet endothelium likely involves the nephrin-mediated signaling cascade, wherein phosphorylation of the tyrosine sites within the intracytoplasmic C terminal domain of nephrin activates mitogen-activated protein kinase p38 and JNK and thereby the transcription factor activating protein-1 (AP-1)/c-Jun, which modulates apoptosis. The study with islet MECs also detected an increased production of the proinflammatory cytokine IL-1β, which can induce Fas expression enabling Fas-mediated apoptosis [31].

8. Conclusion

The relation between diabetic micro- and macroangiopathy and endothelial dysfunction is complex and is still a subject of extensive research. Especially in type 2 diabetes a lot of factors are involved including hyperglycemia, hyperinsulinemia, insulin resistance, dyslipidemia, hypertension, and obesity, which all influence each other and probably intensify each others actions. More insights into the exact mechanisms underlying endothelial dysfunction may lead to important treatment strategies which can significantly reduce the morbidity and mortality rate caused by endothelial dysfunction especially in diabetes patients. Although apoptosis is a natural phenomenon in all multicellular organisms, an increased and accelerated rate of apoptosis of endothelial cells is probably a crucial factor in diabetic comorbidity. There are many pathways involved in activating endothelial cell apoptosis and all of these pathways can be activated in multiple ways. A common mechanism causing endothelial dysfunction and endothelial cell apoptosis is oxidative stress. Several studies show contradictory results regarding a possible role for antioxidants in the treatment to prevent micro- and macroangiopathy. However a treatment aimed at reducing oxidative stress in endothelial cells may be an answer to this major problem, especially since diabetes will soon become an even bigger health problem involving more than 5% of the world population.

Abbreviations

ACE: Angiotensin Converting Enzyme
AGE: Advanced Glycation End-products
AIF: Apoptosis Inducing Factor
ANG-II: Angiotensin II
AMP: Adenosine Monophosphate
AMPK: AMP-activated Protein Kinases
APAF-1: Apoptotic Peptidase Activating Factor 1
ARB: Angiotensin II type 1 Receptor Blocker
ASK-1: Apoptosis Signal Regulating Kinase 1
BMI: Body Mass Index
cGMP: Cyclic Guanosine MonoPhosphate
COX: Cyclooxygenase
CRP: C-Reactive Protein
CVD: Cardio Vascular Disease
DISC: Death Inducing Signaling Complex
DNA: Deoxyribonucleic Acid
Egr-1: Early Growth Response Protein 1
EMP: Endothelial Micro Particle
ERK: Extra cellular signal-Regulated Kinase
ET-1: Endothelin-1
FADD: Fas Associated Death Domain
FasL: Fas-ligand or CD95-ligand
FFA: Free Fatty Acids
FOXO-1: Forkhead box O1
GLUT-4: Glucose Transporter-4
GADP: Glyceraldehyde 3-Phosphate
GSH: Gluthathione
HbA1c: Hemoglobin A1c
HDL: High Density Lipoproteins
HUVEC: Human Umbilical Vein Endothelial Cell
ICAD: Inactive Caspase Activated DNase
ICAM: Intracellular Adhesion Molecule
IL-6: Interleukine-6
IR: Insulin Receptor
IRS-1: Insulin Receptor-Substrate-1
IGT: Impaired Glucose Tolerance
JNK: c-Jun NH 2-Terminal Kinase
LAM: Leucocyte Adhesion Molecule
MAPK: Mitogen-Activated Protein Kinase
MCP-1: Monocyte Chemotactic Protein-1
MEC: Microvascular Endothelial Cell
MEKK1: Methyl Ethyl Ketone Kinase-1
MP: Micro Particle
mRNA: messenger Ribonucleic Acid
NADP(H): Nicotinamide adenine dinucleotide phosphate
NEFA: Non-Esterified Fatty Acid
NO: Nitric Oxide
NOS: Nitric Oxide Synthase
NF-κB: Nuclear Factor κB
OPG: Osteoprogesterin
PARP: Poly ADP-Ribose Polymerase

PAI-1: Plasminogen Activator Inhibitor-1
PMN: Polymorphonuclear Leukocyte
PTP: Permeability Transition Pore
PDGF: Platelet-Derived Growth Factor
PGE2: Prostaglandin E2
PGI2: Prostacyclin
PI-3K: Phosphoinositide-3 Kinase
ROS: Reactive Oxygen Species
SAβ-gal: β-Galactosidase
SAPK: Stress-Activated Protein Kinases
SOD: Superoxide Dismutase
STZ: Streptozotocin
T1DM: Type 1 Diabetes Mellitus
T2DM: Type 2 Diabetes Mellitus
TNFα: Tumor Necrosis Factor-α
t-PA: Tissue-type Plasminogen Activator
TRAIL: TNF-Related apoptosis Inducing Ligand
VCAM: Vascular Cell Adhesion Molecule
VEGF: Vascular Endothelial Growth Factor
VLDL: Very Low Density Lipoproteins
VSMC/VSM: Vascular Smooth Muscle (Cell)
vWF: Von Willebrand Factor.

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Research Article

Leptin Inhibits the Proliferation of Vascular Smooth Muscle Cells Induced by Angiotensin II through Nitric Oxide-Dependent Mechanisms

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Objective. This study was designed to investigate whether leptin modifies angiotensin (Ang) II-induced proliferation of aortic vascular smooth muscle cells (VSMCs) from 10-week-old male Wistar and spontaneously hypertensive rats (SHR), and the possible role of nitric oxide (NO). *Methods*. NO and NO synthase (NOS) activity were assessed by the Griess and ^{3}H-arginine/citrulline conversion assays, respectively. Inducible NOS (iNOS) and NADPH oxidase subutnit Nox2 expression was determined by Western-blot. The proliferative responses to Ang II were evaluated through enzymatic methods. *Results*. Leptin inhibited the Ang II-induced proliferative response of VSMCs from control rats. This inhibitory effect of leptin was abolished by NOS inhibitor, NMMA, and iNOS selective inhibitor, L-NIL, and was not observed in leptin receptor-deficient *fa/fa* rats. SHR showed increased serum leptin concentrations and lipid peroxidation. Despite a similar leptin-induced iNOS up-regulation, VSMCs from SHR showed an impaired NOS activity and NO production induced by leptin, and an increased basal Nox2 expression. The inhibitory effect of leptin on Ang II-induced VSMC proliferation was attenuated. *Conclusion*. Leptin blocks the proliferative response to Ang II through NO-dependent mechanisms. The attenuation of this inhibitory effect of leptin in spontaneous hypertension appears to be due to a reduced NO bioavailability in VSMCs.

1. Introduction

Hypertension is associated with structural changes in blood vessels known as "vascular remodelling" that include an altered proliferation, hypertrophy, migration, and apoptosis of vascular smooth muscle cells (VSMCs), together with an increased extracellular matrix abundance [1]. Angiotensin (Ang II) constitutes one of the main factors involved in vascular remodelling during the onset of hypertension [1]. Angiotensin II exerts pleiotropic actions on the vasculature, such as vasoconstriction, VSMC migration, proliferation and hypertrophy, increased extracellular matrix formation, and activation of NAD(P)H oxidases [1, 2]. Through these actions Ang II promotes vascular inflammation as well as endothelial dysfunction and structural remodelling.

Leptin, the obesity gene (*ob*) product, participates in the control of body weight by regulating food intake and energy expenditure [3, 4]. In addition to the maintenance of energy homeostasis, leptin induces a balanced effect on the control of blood pressure (BP) with a pressor response attributable to sympathetic activation via the central nervous system and a depressor response due to a direct effect of leptin on peripheral tissues [5]. Leptin increases the vasomotor sympathetic activity through the activation of leptin receptors (OB-R) in the ventromedial and dorsomedial hypothalamic regions [6]. On the other hand, leptin exerts a direct vasodilation

through different mechanisms, which include the release of endothelial nitric oxide (NO) in the aorta and coronary arteries [7–9] and endothelium-derived hyperpolarizing factor (EDHF) in mesenteric arteries [8, 10], as well as the inhibition of the Ang II-induced calcium increase and vasoconstriction in the smooth muscle layer of the aorta via NO [11]. A further mechanism whereby leptin decreases BP is related to the induction of natriuresis and diuresis at the tubular level through NO-dependent mechanisms [12, 13]. Increased circulating concentrations of leptin are found in hypertensive animal models [14, 15] and humans [16, 17], suggesting a possible link between hyperleptinemia, and cardiovascular dysfunction in hypertension. In this respect, it has recently been reported that the beneficial vascular, renal and cardiac responses induced by leptin are impaired in hypertensive rats [10, 12, 14, 15].

Leptin has been suggested to participate in vascular remodelling, since it induces the proliferation of rat aortic VSMCs [18] and promotes neointimal growth of VSMCs after injury in mice [19]. Nonetheless, these data are not univocal, given that other authors have reported that leptin inhibits cell growth of human VSMCs [20]. These contradictory observations raise some doubts as regards the potential involvement of leptin in vascular remodelling. The present study was designed to examine the effect of leptin on basal and Ang II-induced proliferation of aortic VSMCs obtained from normotensive Wistar rats and age-matched, spontaneously hypertensive rats (SHR). Some experiments were performed upon VSMCs obtained from Zucker *fa/fa* rats to confirm whether the effects of leptin are mediated via OB-R. To gain further insight into the potential role of NO in the proliferative response induced by leptin, the effect of leptin on NO production, NO synthase (NOS) activity and inducible NOS (iNOS) expression was measured directly in VSMCs. Moreover, to further corroborate the participation of NO in the vascular actions of leptin, the effect of the N^G-monomethyl-L-arginine (NMMA), a nonselective inhibitor of NOS, and L-N^6-(1-iminoethyl)-lysine (L-NIL), a selective inhibitor of iNOS, on the inhibitory effect of leptin on the Ang II-induced proliferation of VSMC of the aorta was analyzed.

2. Materials and Methods

2.1. Animals. Age-matched (10-week-old) male normotensive Wistar (breading house of the University of Navarra), SHR and leptin receptor-deficient Zucker *fa/fa* rats (Harlan, Barcelona, Spain) were used in the present study. Rats were maintained under controlled conditions of room temperature (RT) (20 ± 2°C), relative humidity (50 ± 10%), ventilation (at least 15 complete changes of air/h), and artificial light-dark cycle (lights on from 08:00 a.m.–08:00 p.m.). Animals had free access to tap water and fed *ad libitum* with an isoenergetic (13.39 MJ/kg), isoproteic (14%) rodent maintenance diet containing 0.13% sodium (2014S Teklad Global 14% Protein Rodent Maintenance Diet, Harlan). All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (Directive 86/609/EEC) and were approved by the Ethical Committee for Animal Experimentation of the University of Navarra (036/03). After regular overnight feeding, rats were sacrificed by decapitation in a nonfasted state, since fasting has been shown to reduce circulating concentrations of leptin [4]. Blood samples were immediately collected, and sera were obtained by cold centrifugation (4°C) at 700 *g* for 15 minutes. The thoracic aorta was carefully excised, dissected out, and processed for each study.

2.2. Blood Measurements. Serum glucose concentrations were measured using a sensitive-automatic glucose sensor (Ascensia Elite, Bayer, Barcelona, Spain). Serum concentrations of triglycerides, total cholesterol (Infinity, Thermo Electron Corporation, Melbourne, Australia), and free fatty acids (FFA) (WAKO Chemicals, GmbH, Neuss, Germany) were measured by enzymatic methods, using available commercial kits. Insulin and leptin were determined by ELISA (Crystal Chem, Inc., Chicago, IL, USA). Intra- and interassay coefficients of variation for measurements of insulin and leptin were 3.5% and 6.3%, respectively, for the former, and 5.4% and 6.9%, for the latter. Lipid peroxidation, as an indicator of oxidative stress, was estimated by the measurement of thiobarbituric acid reactive substances (TBARS) in serum as previously described by Conti et al. [21] with some modifications. Serum malondialdehyde (MDA), the best-known specific TBARS, was used as indicator of lipid peroxidation and oxidative stress. Five μL of serum samples or standard MDA (Sigma, St. Louis, MO, USA) were mixed with 120 μL of diethyl thiobarbituric acid (DETBA) 10 mmol/L and vortexed for 5 seconds. The reaction mixture was then incubated at 95°C for 60 minutes. After cooling to room temperature (RT) for 5 minutes, DETBA-MDA adducts were extracted in 360 μL *n*-butanol (Panreac, Barcelona, Spain) vortexing for 1 minute and centrifuged at 1,600 *g* for 10 minutes at RT. Then, the chromophore of the DETBA-MDA adduct was quantified in 200 μL of the upper butanol phase by fluorescence emission at 535 nm with an excitation at 590 nm. MDA equivalents (TBARS) were quantified using a calibration curve prepared using MDA standard working solutions.

2.3. Isolation of Vascular Smooth Muscle Cells. Primary VSMCs were obtained from the thoracic aorta by the tissue explants method, as previously described [11, 15]. Briefly, the smooth muscle tissue was longitudinally opened and cut in small pieces that were grown in plastic 6-well plates and maintained at 37°C in a humidified incubator with an atmosphere of 95% air, 5% CO_2. Tissue explants were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) (Life Technologies, Inc., Gaithersburg, MD, USA) and antibiotic-antimycotic products (10,000 U/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B as Fungizone in 0.85% saline) (Life Technologies). The medium was changed initially after 24 hours, and then every 2-3 days. After about 8–10 days, when cells had formed a confluent monolayer, they were harvested by addition of 0.05% trypsin,

and the culture was continued up to 4–6 passages using DMEM containing 10% FBS.

2.4. Cell Proliferation Assay. Cell proliferation of VSMCs was measured using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Charbonnier, France), according to the manufacturer's instructions. VSMCs were plated in 96-well plate (3,500 cell per well) and incubated for 24 hours in DMEM containing 10% FBS. Quiescence was induced by incubating the cells in DMEM containing 0.1% FBS for 48 hours. Serum-deprived VSMCs were stimulated for 72 hours with different concentrations of Ang II (0.1–1,000 nmol/L) (Sigma) in order to obtain a concentration-response curve for the determination of the pD2 value. In a second subset of experiments, cells were incubated with different concentrations of leptin (0.1–100 nmol/L) (PreproTech EC, Inc., Rocky Hill, NJ, USA) for 72 hours in the absence or presence of Ang II (100 nmol/L). In a third subset of experiments, cells were stimulated with leptin (10 nmol/L) for 72 hours in the presence of Ang II (100 nmol/L) and NMMA (10 μmol/L) (Sigma) or L-NIL (10 μmol/L) (Sigma). The concentration of leptin as well as the pharmacological NOS inhibitors to carry out the experiments was chosen on the basis of prior experiments performed in our laboratory [11, 15]. Following the cell treatment, 20 μL of CellTiter 96 Aqueous One Solution were added to each well and the plate was incubated in the darkness at 37°C for 4 hours. Optical densities were measured at 490 nm using a microplate reader (Sunrise, Tecan, Germany). Proliferative values were expressed as percentage of proliferation of treated cells compared to basal proliferation of unstimulated cells.

2.5. Western-Blot Analyses. Quiescent VSMCs were stimulated for 30 minutes with leptin (10 nmol/L). At different times of the stimulation (0, 10, 20, and 30 minutes), cells were harvested and homogenized in ice-cold lysis buffer (0.1% SDS, 1% Triton X-100, 5 mM EDTA·$2H_2O$, 1 M Tris-HCl, 150 mM NaCl, 1% sodium deoxycholate, pH 7.40) supplemented with a protease inhibitor cocktail (Complete Mini-EDTA free, Roche, Mannheim, Germany). Lysates were centrifuged at 16,000 *g* at 4°C for 15 minutes. Total protein concentrations were determined by the Bradford assay [22], using bovine serum albumin (BSA) (Sigma) as standard [23]. Thirty micrograms of total protein were diluted in loading buffer 4X (20% β-mercaptoethanol, 40 mmol/L dithiothreitol, 8% SDS, 40% glycerol, 0.016% bromophenol blue, 200 mmol/L Tris-HCl, pH 6.80) and heated for 10 minutes at 100°C. Samples were run out in 8% SDS-PAGE, subsequently transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked in Tris-buffered saline (10 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.00) with 0.05% Tween 20 (TBS-T) containing 5% nonfat dry milk for 1 hour at RT. Blots were then incubated overnight at 4°C with rabbit polyclonal anti-Akt1, rabbit polyclonal anti-phospho-(Thr^{308})-Akt (Upstate, Lake Placid, NY, USA), rabbit polyclonal anti-STAT3, rabbit polyclonal anti-phospho-(Tyr^{705})-STAT3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse monoclonal anti-iNOS (BD Transduction Laboratories, San Jose, CA, USA), rabbit polyclonal anti Nox2/gp91phox (Abcam, Cambridge, UK), or murine monoclonal anti-β-actin (Sigma) antibodies. The antigen-antibody complexes were visualized using peroxidase-conjugated antirabbit or antimouse antibodies (1 : 5,000) and the enhanced chemiluminescence ECL detection system (Amersham Biosciences, Buckinghamshire, UK). The intensity of the bands was determined by densitometric analysis and normalised with β-actin density values.

2.6. Evaluation of NO Production and NOS Activity. Quiescent VSMCs were stimulated during 30 minutes with leptin (10 nmol/L) in the presence or absence of NMMA (10 μmol/L) or L-NIL (10 μmol/L). One sample per assay was used to obtain control responses in the presence of solvent. Samples of the culture media were collected at different times (0, 10, 20 and 30 minutes) for the measurement of nitrates and nitrites ($[NO_x]$), as an index of NO production, with a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) based on the Griess reaction following the manufacturer's protocol. The intra- and inter-assay coefficients of variation were 3.3% and 6.5%, respectively. Stimulated cells were harvested and homogenised in a lysis buffer (25 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L EGTA; pH 7.40) supplemented with a protease inhibitor cocktail (Roche) for the determination of NOS activity. The protein content of the homogenates was determined by the method of Bradford [22]. NOS activity was measured by the L-[^{3}H]arginine to L-[^{3}H]citrulline conversion assay, using a commercial kit (Stratagene, La Jolla, CA, USA). The intra- and inter-assay coefficients of variation were 6.3% and 9.1%, respectively. Briefly, samples of 20 μg of protein were incubated at room temperature (RT) for 1 hour in the reaction buffer [25 mmol/L Tris-HCl (pH 7.40), 3 μmol/L tetrahydrobiopterin, 1 μmol/L FADH, 1 μmol/L $FMNH_2$, 1 mmol/L NADPH, 0.6 mmol/L $CaCl_2$] supplemented with L-[^{3}H]arginine (1 μCi/μL) (Amersham Biosciences). L-[^{3}H]citrulline was quantified by using a scintillation counter (Wallac 1409 DSA, PerkinElmer, Inc., Barcelona, Spain). All assays were performed in duplicate.

2.7. Statistical Analysis. Data are presented as mean ± standard error of the mean (SEM). Concentration-response curves were fitted by nonlinear regression, the concentration giving 50% of the maximal response (EC_{50}) was determined, and the pD_2 was calculated as $-\log EC_{50}$ (mol/L). Statistical differences among mean values were determined using the two-way ANOVA, one-way ANOVA followed by Dunnett's *t* test, or the Student's *t* test, where appropriate. A P value $< .05$ was considered statistically significant. Analyses were performed by the SPSS/Windows version 15.0.1 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Metabolic Profile and Serum Leptin Concentrations. General characteristics of the carbohydrate and lipid metabolism of experimental animals are shown in Table 1. SHR were

Table 1: Metabolic characteristics of normotensive and hypertensive animals.

Determination	Wistar rats ($n = 14$)	SHR ($n = 28$)	P value
Body weight (g)	283.6 ± 9.4	303.3 ± 2.6	**.001**
Free fatty acids (mg/dL)	20.8 ± 1.7	20.3 ± 0.9	.776
Triglycerides (mg/dL)	105.3 ± 19.2	129.5 ± 6.7	**.05**
Total cholesterol (mg/dL)	113.5 ± 6.4	134.1 ± 3.2	**.002**
Glucose (mg/dL)	133.4 ± 0.7	205.5 ± 0.5	**.002**
Insulin (ng/mL)	1.3 ± 0.3	3.0 ± 0.3	**.001**
Leptin (ng/mL)	2.5 ± 0.1	3.1 ± 0.1	**.05**
TBARS (μmol/L)	1.4 ± 0.2	2.2 ± 0.2	**.025**

SHR, spontaneously hypertensive rats; TBARS, thiobarbituric acid reactive substances. Values presented as the mean ± SEM. Differences between groups were analysed by Student's t-test. Bold values are statistically significant P values among groups.

heavier ($P < .001$) and exhibited higher serum glucose ($P < .01$) and insulin ($P < .001$) concentrations than age-matched Wistar rats. Serum triglycerides and total cholesterol were also increased ($P < .05$ and $P < .01$, resp.) in SHR, compared to Wistar rats. The circulating concentrations of leptin were increased ($P < .05$) in the SHR group. A positive correlation between serum leptin levels and body weight ($r = 0.67$, $P < .0001$) was found. The serum levels of TBARS, as the index of oxidative stress, were significantly ($P < .05$) increased in SHR compared to control rats.

3.2. Effect of Leptin on Ang II-Induced Proliferative Response in VSMCs. Ang II elicited a concentration-dependent ($P < .00001$) increase in the proliferation of aortic VSMCs obtained from Wistar rats ($pD_2 = 9.1 \pm 0.6$) (Figure 1). A concentration of Ang II 100 nmol/L, inducing a proliferative response of 193 ± 17% compared to basal proliferation, was chosen for subsequent experiments.

All the tested leptin concentrations significantly inhibited ($P < .05$) the basal proliferation of aortic VSMCs from Wistar rats (Figure 2(a)). Moreover, leptin induced a decrease ($P < .01$) in Ang II-induced proliferative response in VSMCs from Wistar rats (Figure 2(b)). To test that the inhibitory effect of leptin is mediated via its binding to leptin receptors, the experiments were also performed in VSMCs obtained from Zucker *fa/fa* rats, a genetic model of leptin receptor resistance. As earlier reported by other authors [24, 25], Zucker *fa/fa* rats were severely obese, and showed hyperglycaemia, hyperinsulinemia, hyperlipidemia, and hyperleptinemia (Table 2). No inhibitory effect of leptin ($P = .409$) was observed on Ang II-induced proliferation in VSMCs obtained from the aorta of Zucker *fa/fa* rats (Figure 2(c)).

To determine whether this vascular action of leptin may be altered in hypertension, we assessed the effect of leptin on Ang II-induced proliferative response in aortic VSMCs from SHR rats. Although leptin was able to inhibit ($P < .01$) the Ang II-induced proliferation in VSMCs from SHR (Figure 2(d)), the reduction of the response to Ang

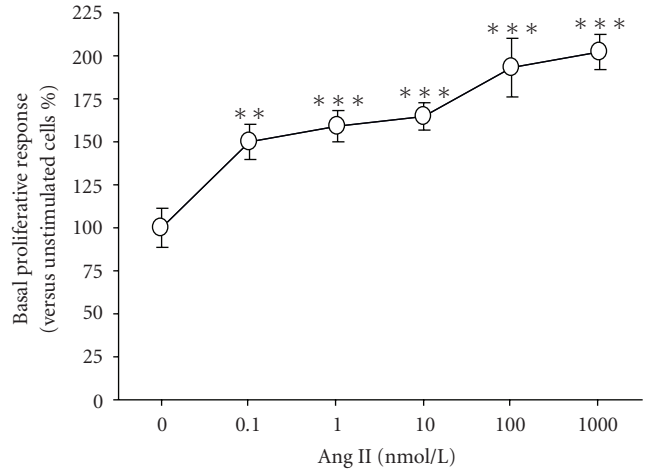

Figure 1: Concentration-response curve of the proliferation induced by angiotensin (Ang) II in aortic vascular smooth muscle cells (VSMCs) obtained from Wistar rats. Values are the mean ± SEM (n = 10–15). Differences between groups were analysed by one-way ANOVA followed by Dunnet's test. $^{**}P < .01$, $^{***}P < .001$ versus control response in unstimulated cells.

Table 2: Metabolic characteristics of Zucker *fa/fa* rats.

Determination	Wistar rats ($n = 14$)	Zucker *fa/fa* rats ($n = 10$)	P value
Body weight (g)	283.6 ± 9.4	403.1 ± 4.9	**.00001**
Free fatty acids (mg/dL)	20.8 ± 1.7	15.7 ± 2.1	.081
Triglycerides (mg/dL)	105.3 ± 19.2	285.1 ± 16.6	**.00001**
Total cholesterol (mg/dL)	113.5 ± 6.4	131.4 ± 2.1	**.05**
Glucose (mg/dL)	133.4 ± 0.7	166.8 ± 1.1	**.05**
Insulin (ng/mL)	1.3 ± 0.3	10.8 ± 0.4	**.00001**
Leptin (ng/mL)	2.5 ± 0.1	49.8 ± 0.3	**.00001**

Values presented as the mean ± SEM. Differences between groups were analysed by Student's t test. Bold values are statistically significant P values among groups.

II was lower than that of control Wistar rats in all tested concentrations of leptin (0.1 nmol/L, 18 ± 6% versus 28 ± 4%; 1 nmol/L, 17 ± 5% versus 28 ± 3% versus 17 ± 5%; 10 nmol/L, 15 ± 6% versus 31 ± 3%; 100 nmol/l, 41 ± 2% versus 24 ± 8%, resp.).

3.3. Effect of Leptin on Ang II-Induced Proliferation of VSMCs in the Presence of NOS Inhibitors. Our group previously described that leptin induces the synthesis of NO through the activation of iNOS in VSMCs [11]. The effect of leptin on the Ang II-induced proliferative response of aortic VSMCs obtained from Wistar rats was reexamined in the presence of the NOS inhibitor, NMMA, or the iNOS selective inhibitor, L-NIL. The concentration of leptin 10 nmol/L, reducing by ~15% the basal proliferation and by ~30% the Ang II-induced proliferation in aortic VSMCs, was chosen to carry out these experiments. Both NOS inhibitors completely abolished the inhibitory effect of leptin on the Ang II-mediated proliferation (Figure 3). Moreover, the presence of

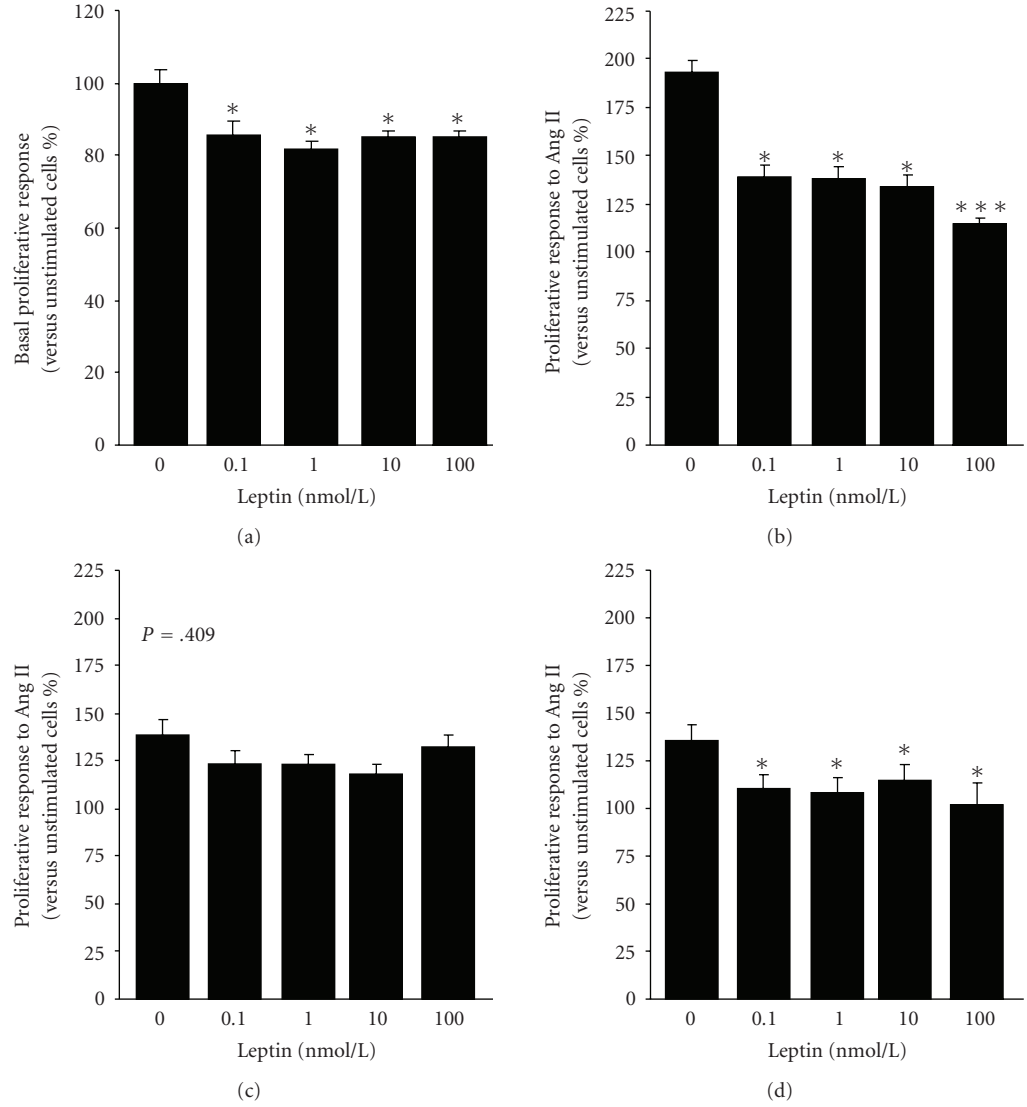

Figure 2: Effect of leptin on basal and Ang II-induced proliferation of aortic VSMCs. Aortic VSMCs obtained from Wistar rats were incubated for 72 hours with increasing concentrations of leptin (0.1–100 nmol/L) in the absence (a) or presence (b) of Ang II (100 nmol/l), and the proliferative response was measured using a tetrazolium dye (MTT)-based proliferation assay. Effect of leptin on Ang II (100 nmol/l)-induced proliferation in VSMCs obtained from the aorta of leptin receptor-deficient Zucker *fa/fa* rats (c) and spontaneously hypertensive rats (SHR). Values are the mean ± SEM ($n = 40$). Differences between groups were analysed by one-way ANOVA followed by Dunnet's test. $^{*}P < .05$, $^{***}P < .001$ versus control response in unstimulated cells (a) or to Ang II (b, c).

NMMA or L-NIL blunted the inhibition of basal proliferation induced by leptin (105 ± 1% and 107 ± 2% versus 85 ± 2% mg, resp.). Basal and Ang II-induced proliferation of aortic VSMCs was not affected by the presence of NOS inhibitors.

3.4. Impaired NOS Activity and NO Production in VSMCs in Hypertensive Rats. The activation of the JAK2/STAT3 and PI3K/Akt pathways constitutes an early step for the up-regulation of iNOS induced by leptin [11, 26, 27]. The ability of leptin to trigger JAK2/STAT3 and PI3K/Akt was examined by the degree of phosphorylation/activation of the downstream molecules STAT3 and Akt after leptin treatment in VSMCs from Wistar rats and SHR. Leptin activated the phosphorylation of STAT3 in a time-dependent manner, whereas a maximal phosphorylation of Akt was observed after 10 minutes of leptin stimulation with attenuation of the phosphorylation thereafter (Figures 4(a) and 4(b)). No differences between VSMCs from Wistar and SHR were found for the activation/phosphorylation of Akt and STAT3. Accordingly, leptin induced a significant increase in iNOS expression in aortic VSMCs from Wistar and SHR (Figure 4(c)). Nevertheless, the ability of leptin to induce NO production and NOS activity was impaired in aortic VSMCs obtained from SHR (Figures 5(a) and 5(b)). It is well known that enhanced production of NO-scavenger substances such as reactive oxygen species (ROS)

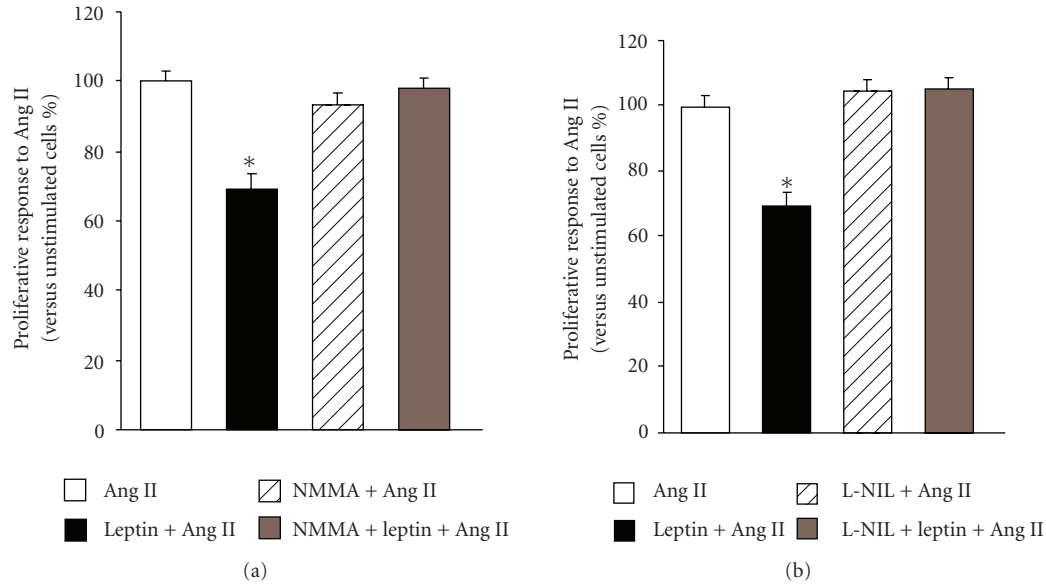

Figure 3: Impact of NOS inhibitors on the inhibitory effect of leptin on Ang II-induced proliferation of aortic VSMCs. The coincubation with both NOS inhibitor, NMMA (10 μmol/l), (a) and the selective iNOS inhibitor, L-NIL (10 μmol/l), (b) blunted the inhibitory effect of leptin (10 nmol/l) on the Ang II (100 nmol/l)-induced proliferative response in aortic vascular smooth muscle cells (VSMCs) from Wistar rats. Data are expressed as mean ± SEM (n=40). Differences between groups were analysed by two-way ANOVA. In case of interaction between factors (leptin treatment and NOS inhibitors), differences between groups were analysed by one-way ANOVA followed by Dunnet's test. $^{*}P < .05$, $^{**}P < .01$ versus control response to Ang II in the absence of inhibitors.

under spontaneous hypertension is involved in reducing NO bioavailability [2]. Thus, we compared the basal expression of Nox2, a subunit of the ROS-generating NADPH oxidase, in VSMCs from control Wistar rats and SHR. The protein levels of Nox2 were significantly ($P < .05$) increased in VSMCs from hypertensive rats (Figure 5(c)).

4. Discussion

The smooth muscle layer represents an important target for the vascular effects of leptin [11, 15]. This adipokine decreases passive wall tension and Ang II-induced vasoconstriction operating directly on VSMCs [11]. Despite the growing evidence supporting the depressor action of leptin on blood vessels, the role of leptin on vascular remodelling remains unclear [18–20]. Thus, the present study has further explored the mechanisms whereby leptin participates in the proliferation of VSMCs, a crucial process involved in vascular remodelling.

Our results show that leptin inhibits the basal proliferation of aortic VSMCs in Wistar rats, which is in concordance with findings reported by Bohlen and colleagues using human aortic VSMCs [20]. Moreover, we show, for the first time, that leptin inhibits the Ang II-induced cell growth of VSMCs. To test directly whether this inhibitory effect is dependent on leptin signalling, the experiments were also performed in VSMCs obtained from the aorta of Zucker *fa/fa* rats, an animal model with a missense mutation in the leptin receptor gene (OB-$R^{269gln \rightarrow pro}$) that results in both a reduced affinity for leptin and reduced signal transduction capability [24, 25]. As a result of this genetic leptin receptor resistance, Zucker rats show severe metabolic alterations, including severe obesity, hyperglycemia, hyperinsulinemia, insulin resistance, and hypogonadism [24, 25]. This animal model of leptin resistance, that is, the obese Zucker *fa/fa* rats, also shows hypogonadism that further aggravates the obese phenotype, since leptin can regulate the expression and secretion of gonadotropins, and the hypothalamic-pituitary-gonadal axis is closely associated to food intake, body weight, and fat distribution [28]. In the present study, our findings showed the lack of effect of leptin on Ang II-induced proliferation in aortic VSMCs from Zucker rats, suggesting that functional leptin receptors are required for this vascular effect of the hormone.

A functional relation between leptin and NO has been established in blood vessels [7, 8, 11, 29]. Frühbeck showed that intravenous administration of leptin in rats with autonomic blockade induces a systemic vasodilation that is associated with an increase of serum [NO_x] and reversed with Nω-nitro-L-arginine methyl ester [7]. Further studies have shown that leptin induces an endothelial-dependent vasodilation by activating a PI 3-kinase-independent Akt-endothelial NOS (eNOS) phosphorylation pathway [29, 30]. Moreover, leptin treatment in vivo has been shown to reverse the endothelial dysfunction of leptin-deficient obese (*ob/ob*) mice by increasing NO bioavailability in vessels [31]. This adipokine decreases passive wall tension and Ang II-induced vasoconstriction by up-regulating iNOS through mechanisms involving JAK2/STAT3 and PI3K/Akt pathways

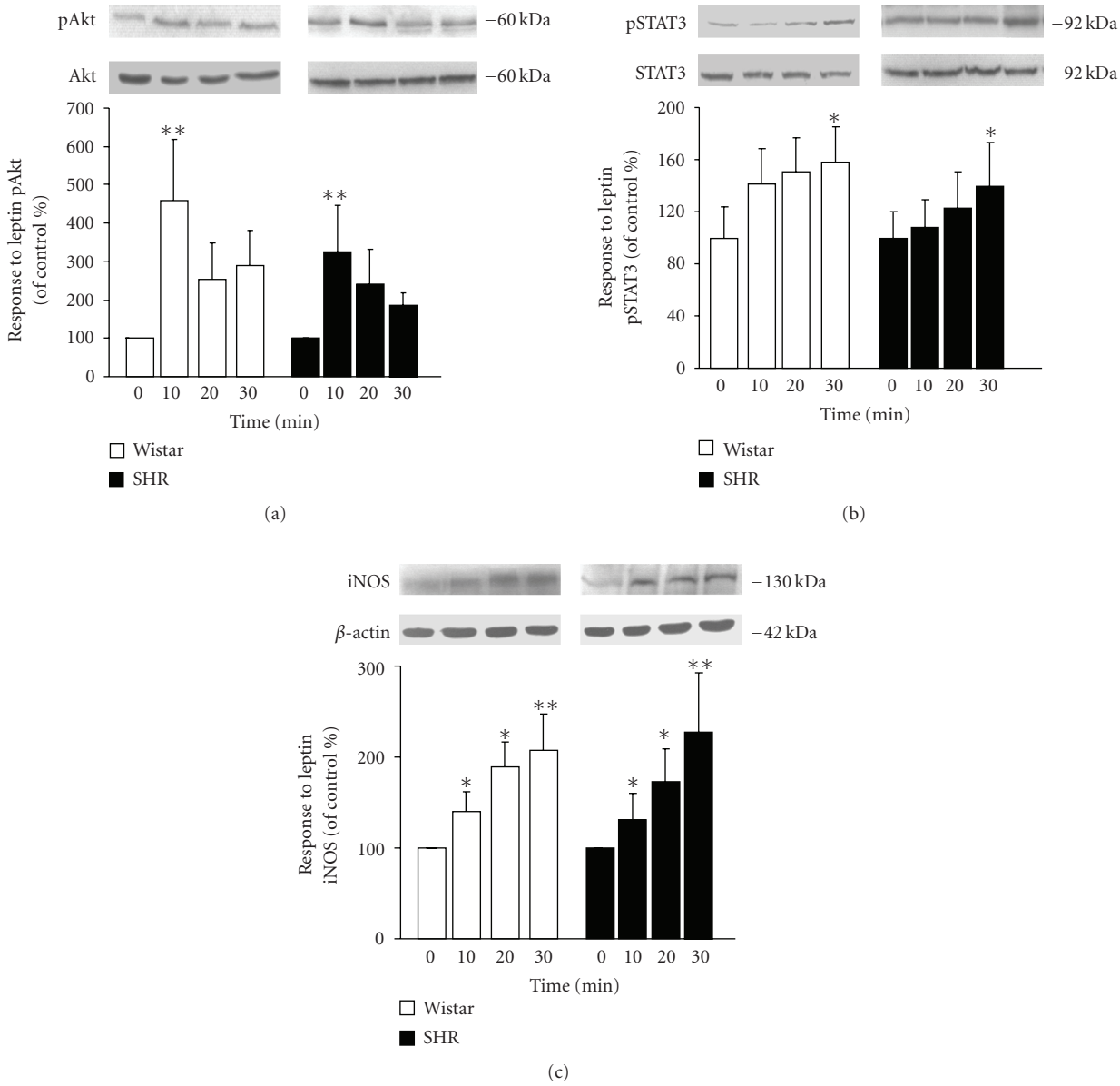

Figure 4: Effect of leptin activation of Akt and STAT3 and iNOS expression in aortic VSMCs. Bar graphs show the differences in the time course of Akt (a) and STAT3 (b) activation/phosphorylation as well as iNOS (c) protein expression in leptin (10 nmol/L)-stimulated aortic VSMCs from Wistar and SHR. Data are expressed as mean ± SEM ($n = 10$). Differences between groups were analysed by two-way ANOVA. In case of interaction between factors (strain and time of leptin stimulation), differences between groups were analysed by one-way ANOVA followed by Dunnet's test $^{*}P < .05$, $^{**}P < .01$ versus unstimulated cells.

in VSMCs [11]. The ability of leptin to induce iNOS gene expression has been shown in several cell types, such as murine J774A.1 macrophages, rat adipocytes, human primary chondrocytes and ATDC5 cells, C6 glioma cell line, and human OA cartilage [26, 27, 32–34]. Our findings showed that the inhibitory effect of leptin on Ang II-induced cell growth of VSMCs is completely prevented by an iNOS inhibitor. Previous data reported by our group provided evidence that the depressor action of leptin in the smooth muscle layer of the aorta takes place by reducing the vasoconstrictor potential of Ang II through NO-dependent mechanisms [11]. Similar findings of hypotensive effects of leptin via NO have been reported in rat myocardium [35], kidneys[13], endothelium of conduit vessels (aorta) [8, 29, 30], and resistance vessels (mesenteric and coronary arteries) [8, 9]. Taken together, these data support the notion that NO represents a key mediator of the cardiovascular effects of leptin.

SHR constitute a well-known model of essential hypertension that becomes hypertensive at an early stage (4–6 weeks of age) [36]. Early vascular remodelling experienced by the aorta of SHR leads to a reduced contractility in vitro and,

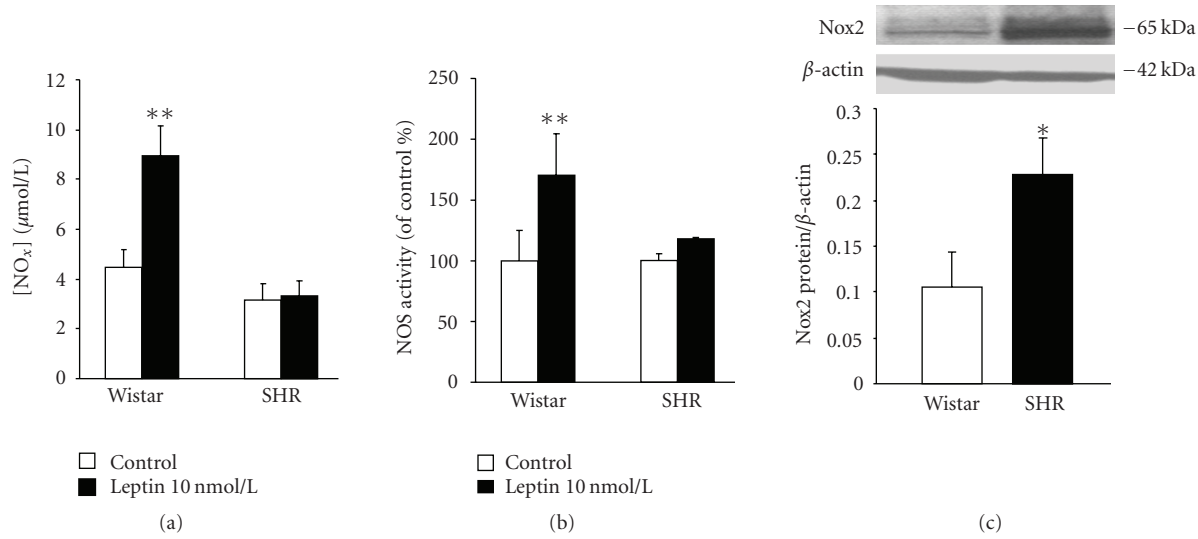

Figure 5: Impaired NO production and NOS activity and increased NADPH oxidase expression in aortic VSMCs from hypertensive rats. Bar graphs show (a) the accumulation of NO_x in the culture media and (b) the NOS activity of aortic VSMCs from control Wistar rats and spontaneously hypertensive rats (SHR) stimulated with leptin (10 nmol/l) for 30 minutes. (c) Basal expression of Nox2, a subunit of the NADPH oxidase, in VSMCs from control Wistar rats and SHR. Data are expressed as mean ± SEM ($n = 10$). Differences between groups were analysed by two-way ANOVA (a, b) or Student's t test (c). In case of interaction between factors (strain and leptin treatment), differences between groups were analysed by one-way ANOVA followed by Dunnet's test $^{*}P < .05$, $^{**}P < .01$ versus control response in unstimulated cells (a, b) or VSMCs from control rats (c).

probably, to vascular rigidity in vivo [2, 15]. Overactivation of the renin-angiotensin system constitutes an important contributor to the vascular remodelling associated with the onset of hypertension in SHR [1]. Touyz and colleagues [37] reported that Ang II concentration dependently increased the ^{3}H tymidine incorporation in VSMCs, as an index of synthesis of DNA and cell proliferation, with enhanced responsiveness in VSMCs from SHR compared to control rats. In the present study, SHR showed features of the human metabolic syndrome, such as overweight, hyperglycaemia, hyperinsulinemia, insulin resistance, dyslipidemia, and increased circulating concentrations of leptin, which confirm data reported by our group and others [12, 38]. Moreover, our results show that aortic VSMCs from SHR are less responsive to the inhibitory effect of leptin on Ang II-induced proliferation. These findings are in agreement with other studies reporting an impairment of the depressor actions of leptin (or leptin resistance) under spontaneous hypertension in rat myocardium [14], mesenteric arteries [10], aorta [15], and kidneys [12]. Interestingly, we found that, despite a similar activation of JAK2/STAT3 and PI3K/Akt and increased expression of iNOS than that observed in normotensive rats, VSMCs from SHR showed an impaired NOS activity and NO production induced by leptin as well as higher basal Nox2 expression, a subunit of the reactive oxygen species (ROS)-producing NADPH oxidases. It is well known that endothelial dysfunction in SHR is characterized by a reduced synthesis and release of endothelium-derived relaxing factors, such as NO and/or an enhanced production of reactive oxygen species (ROS), which scavenge NO within vessels to reduce its biological half-life [2]. In VSMCs, Ang II reportedly increased the ROS-generating enzymes NADPH oxidases and ROS function as important intra- and intercellular second messengers to modulate many downstream signalling molecules, such as protein tyrosine phosphatases (PTPs), protein tyrosine kinases, transcription factors, mitogen-activated protein kinases (MAPKs), and ion channels, leading to VSMCs growth and migration [2]. In the present study, our data showed an increased systemic oxidative stress as well as higher expression levels in VSMCs of Nox2 in hypertensive rats. Together, it could be speculated that, in the setting of hypertension, the antiproliferative effects of leptin are overridden by the effects of Ang II, despite the hyperleptinemia. Among the different mechanisms that may underlie this finding, the role of Ang II-induced production of ROS could be important in experimental hypertension.

In conclusion, our results provide evidence that leptin constitutes a negative modulator of vascular remodelling. This statement is supported by findings reported herein: (a) leptin inhibits the basal and Ang II-induced proliferative response of VSMCs through NO-dependent mechanisms; (b) the lack of effect of leptin on Ang II-stimulated proliferation in VSMCs obtained from leptin receptor-deficient Zucker *fa/fa* rats provides evidence that functional leptin receptors (OB-R) are required for this inhibition; (c) the impairment of the inhibitory effect of leptin on Ang II-induced proliferation of VSMC from SHR appears to be a consequence of a reduced NO biodisponibility due to an increased expression of NADPH oxidases. Therefore, hyperleptinemia may arise as a compensatory mechanism to overcome vascular leptin resistance in SHR.

Acknowledgments

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Research Article

Inflammatory Markers in Middle-Aged Obese Subjects: Does Obstructive Sleep Apnea Syndrome Play a Role?

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Background. Obstructive Sleep Apnea Syndrome (OSAS) is associated with inflammation, but obesity may be a confounding factor. Thus, the aim of this study was to explore differences in serum levels of inflammation markers between obese individuals with or without OSAS. *Methods*. Healthy individuals ($n = 61$) from an outpatient obesity clinic were examined by polysomnography and blood analysis, for measurement of TNF-α, IL-6, CRP, and fibrinogen levels. According to Apnea-Hypopnea Index (AHI), participants were divided into two BMI-matched groups: controls (AHI < 15/h, $n = 23$) and OSAS patients (AHI ≥ 15/h, $n = 38$). *Results*. OSAS patients had significantly higher TNF-α levels ($P < .001$) while no other difference in the examined inflammation markers was recorded between groups. Overall, TNF-α levels were correlated with neck circumference ($P < .001$), AHI ($P = .002$), and Oxygen Desaturation Index ($P = .002$). *Conclusions*. Obese OSAS patients have elevated TNF-α levels compared to BMI-matched controls, suggesting a role of OSAS in promoting inflammation, possibly mediated by TNF-a.

1. Introduction

Obstructive Sleep Apnea Syndrome (OSAS) is a common disorder, known to affect about 4% of middle-aged men and 2% of middle-aged women [1]. Patients exhibit repetitive episodes of partial or complete obstruction of the upper airway during sleep, ultimately leading to increased respiratory effort, oxyhemoglobin desaturation, sleep fragmentation, and excessive daytime sleepiness. Increasing evidence suggests that OSAS is associated with hypertension and other cardiovascular diseases, metabolic derangement, and impaired glucose tolerance [2].

Obesity is not only a well-established risk factor for OSAS [1, 3–6] but also a proinflammatory state [7]. In contrast to earlier theories which considered the adipose tissue as a sole energy depot, current data demonstrate that it is an active endocrine organ, releasing a number of bioactive mediators (adipokines) that modulate blood pressure, lipid- and glucose-metabolism, atherosclerosis, and inflammation [7–9]. Indeed, macrophages of the adipose tissue secrete proinflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) [7, 10].

Similarly, inflammation is one of the postulated links between OSAS and increased cardiovascular morbidity [2]. Indeed, the proinflammatory transcription factor NF-κB is upregulated in OSAS. This is mediated by the alterations between hypoxia and reoxygenation, along with sleep deprivation. NF-κB plays a key role in inflammatory responses, regulating the expression of inflammatory genes [11]. So far, previous studies, as reviewed by Arnardottir et al. [12], have recruited subjects from sleep laboratories, attempting to establish a link between OSAS and inflammation by

focusing on the markers TNF-α, IL-6, and C-reactive protein (CRP). However, the contribution of obesity per se in this inflammatory activity has not been adequately determined [12].

Given that systemic inflammation has been demonstrated in both obesity and OSAS, there may be a possible interaction between them, rendering extremely difficult the distinction between inflammatory processes attributed to either of these two conditions. Therefore, the present study aimed to contribute to the clarification of such issues. Specifically, it attempted to explore the potential differences in four well-established serum inflammation markers (TNF-α, IL-6, CRP, and fibrinogen) between otherwise healthy, obese subjects with OSAS and their nonapneic obese counterparts.

2. Methods

2.1. Subjects. The present study included sixty-one (50 males and 11 females) subjects. These were consecutively recruited from the Outpatient Clinic of Obesity, Diabetes and Metabolism and had consented to be referred for sleep evaluation. None of them had been previously examined or had received treatment for obstructive sleep apnea. Body Mass Index (BMI) was calculated according to the formula BMI = Weight (in kilograms)/[Height (in meters)]^2. All participants had a BMI exceeding 30 kg/m^2.

Exclusion criteria were as follows: known inflammatory or other chronic disease, diabetes mellitus, cardiovascular or cerebrovascular, liver, or endocrine disease, hypertension, chronic use of medication, and smoking. Infection occurring at the time of the examination was an additional exclusion criterion. The study was approved by the institutional ethics committee and all participants had given their informed consent.

2.2. Study Design

2.2.1. Initial Assessment. Medical history was recorded, and physical examination was performed. Anthropometrical data [age, sex, BMI, neck, waist and hip circumference, and waist-to-hip ratio (WHR)] along with daytime habits were recorded. Neck circumference was measured at the cricothyreoid level, waist circumference in the middle between the 12th rib and the iliac crest, and hip circumference at the level of great trochander by a measure tape. Blood pressure was recorded as the average of three repeated measurements in a seated position by an electronic sphygmomanometer adapted to arm circumference. Sleepiness was evaluated by the Greek version of Epworth Sleepiness Scale (ESS) [13].

2.2.2. Polysomnography (PSG). All subjects underwent an attended overnight polysomnography (Somnologica 3.1; Flaga; Reykjavik, Iceland) using a standard montage of electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG), and electrocardiogram (ECG) signals together with pulse oximetry and airflow, detected using combined oronasal thermistors. Thoracic cage and abdominal motion were recorded by inductive plethysmography. Polysomnography was conducted between 22.00 and 06.00 hours. Apneas, hypopneas, and EEG recordings were manually scored according to standard criteria [14]. Apnea Hypopnea Index (AHI) was calculated by dividing the total number of apneas and hypopneas to polysomnographically recorded sleep time, while Oxygen Desaturation Index (ODI) was calculated by dividing the total number of oxyhaemoglobin desaturations $\geq$3% to polysomnographically recorded sleep time.

Two groups were formed, based on AHI in the polysomnographic examination: OSAS patients (AHI $\geq$ 15/hour; n = 38) and controls (AHI < 15/hour; n = 23).

2.2.3. Measurement of Cytokines and Biochemical Analysis. Blood samples were collected between 8 and 9 AM following the polysomnographic examination, while participants were in a fasting state. After blood collection, serum was frozen in aliquots at -80°C immediately after centrifugation (4°C, 1600 g for 15 minutes). TNF-α and IL-6 levels were detected with quantitative sandwich enzyme immunoassay technique (R&D Systems, Minneapolis, USA). Minimum detectable doses of TNF-α and IL-6 were 1.6 pg/mL and 0.7 pg/mL, respectively. High-sensitivity CRP (CRP) was measured by nephelometric method in an image analyzer (Beckmann Coulter; California, USA). Fibrinogen levels were measured by clotting method using a Thrombolyzer B.E. (Behnk Elektronik GmbH) analyzer.

2.2.4. Statistical Analysis. All continuous variables were checked for normality (Kolmogorov-Smirnov test). Descriptive results for continuous variables are expressed as mean $\pm$ SD. Differences between individuals with and without OSAS were examined with independent samples t-test or Mann-Whitney test, and correlations were explored with Pearson's or Spearman correlation, respectively, depending on the normality of data distribution. The reported P-values are two tailed. Significance was defined at the 5% level (P < .05). Analysis was performed using SPSS v.15.0 (SPSS Inc. Chicago, IL).

3. Results

Anthropometric and sleep characteristics of all subjects, as well as the comparison between the two groups (OSAS patients and controls), are presented in Table 1. The two groups were matched in terms of BMI, waist circumference and WHR; however, in OSAS patients a significantly greater mean neck circumference was observed. Indices of lung function, that is, spirometry and arterial blood gases' analysis, were within the normal range in all participants, and blood pressure measurements were below 140/90 mm Hg. As expected, characteristics of respiratory function during sleep in the OSAS group were obviously worse in comparison to the control group.

OSAS patients had significantly higher levels of TNF-α while no difference was detected between the two groups in levels of CRP, IL-6 and fibrinogen (Table 2). Overall, levels of TNF-α were significantly positively correlated with neck circumference (r = 0.452, P < .001), AHI (r = 0.391,

Table 1: Comparison of anthropometric and sleep characteristics between the two groups.

	Total ($n = 61$)	Controls ($n = 23$)	OSAS ($n = 38$)	P-value
Gender (M/F)	50/11	17/6	33/5	.353
Age (years)	44.9 ± 9.2	43.7 ± 6.7	45.5 ± 10.5	.420
BMI (kg/m^2)	35.7 ± 6.3	34.5 ± 3.7	36.4 ± 7.4	.577
Neck circumference (cm)	42.8 ± 3.1	40.5 ± 2	44.1 ± 3	**<.001**
Waist circumference (cm)	117 ± 9.7	114.7 ± 7.9	118.4 ± 10.5	.147
Hip circumference (cm)	118.7 ± 13.7	114.7 ± 8.2	121.2 ± 15.7	.140
WHR	0.99 ± 0.05	1 ± 0.03	0.98 ± 0.06	.169
Systolic BP (mmHg)	131.1 ± 7	131.2 ± 6.4	131.1 ± 7.4	.864
Diastolic BP (mmHg)	80.4 ± 7.9	80.2 ± 7.9	80.6 ± 7.9	.873
FEV_1(% pred)	91.1 ± 9.7	91.2 ± 8.9	91 ± 10.3	.939
FVC (% pred)	89.6 ± 10.2	89 ± 8.8	90 ± 11	.714
PaO_2 (mmHg)	82.4 ± 6.8	82.7 ± 5.8	82.2 ± 7.5	.786
$PaCO_2$ (mmHg)	38.4 ± 2.5	38.6 ± 1.3	38.2 ± 3	.428
pH	7.40 ± 0.02	7.40 ± 0.01	7.41 ± 0.02	.943
ESS	9.9 ± 6.2	6.6 ± 4.6	11.9 ± 6.2	**<.001**
AHI (/hour)	40 ± 34.5	5.3 ± 3.2	61 ± 27	**<.001**
ODI (/hour)	41.9 ± 34.2	7.3 ± 4	62.9 ± 26.4	**<.001**
$avSpO_2$ (%)	90.8 ± 4.6	93.4 ± 1.4	89.3 ± 5.2	**<.001**
$minSpO_2$ (%)	75.7 ± 11.1	84.4 ± 3.1	70.4 ± 10.8	**<.001**
$t < 90$ (% TST)	23.6 ± 27.8	3.3 ± 5	36.3 ± 28.7	**<.001**

Table 2: Comparison of levels of the examined inflammatory markers in the two groups.

	Total ($n = 61$)	Controls ($n = 23$)	OSAS ($n = 38$)	P-value
TNF-α (pg/mL)	5.67 ± 3.32	3.94 ± 1.34	6.72 ± 3.72	**<.001**
CRP (mg/dL)	0.52 ± 0.48	0.47 ± 0.5	0.55 ± 0.47	.125
IL-6 (pg/mL)	2.66 ± 1.19	2.36 ± 1.41	2.73 ± 1.14	.465
Fibrinogen (g/L)	2.63 ± 0.75	2.7 ± 0.65	2.59 ± 0.81	.616

$P = .002$), and ODI ($r = 0.384$, $P = .002$) (Figure 1). Interestingly, there was also a small but statistically significant negative correlation of CRP levels with average SpO_2 ($r = -0.252$, $P = .050$) and minimum SpO_2 ($r = -0.256$, $P = .047$) during sleep. No other correlation between the levels of the examined inflammation markers and anthropometric or sleep characteristics of the recruited subjects was observed.

4. Discussion

This study compared obese OSAS subjects with their non-apneic obese counterparts in terms of four established serum inflammation markers. In comparison to controls matched for BMI, WHR, and waist circumference, higher TNF-α levels were revealed in OSAS patients. TNF-α is an inflammatory cytokine that has been found elevated in patients with sleep apnea [15–17]. It is involved in sleep regulation [18, 19] and has been positively correlated with excessive daytime sleepiness, nocturnal sleep disturbance, and hypoxia [20]. Similar to our findings, Ciftci et al. [15] have reported increased TNF-α levels in the presence of OSAS, and this increase was independent of BMI. However, they studied only males, whom they recruited from a sleep disorders center, instead of an obesity clinic [15]. Elevation of TNF-α has also been observed by Minoguchi et al. [16], but, again, the comparison was between OSAS and obese subjects. Ryan et al. [17] have demonstrated higher TNF-α levels in subjects with than in those without OSAS, but they studied exclusively men and did not examine the impact of obesity on TNF-α elevation. Our study differs from the previous works in two ways. First, we enrolled subjects from an outpatient clinic of obesity, diabetes, and metabolism. Secondly, all our subjects were obese. This enabled us to address (for the first time, to the best of our knowledge) the interplay between obesity and OSAS in the obesity clinic, by comparing OSAS subjects with their non-apneic counterparts. Hence, our new message is that TNF-α is increased in the presence of OSAS among obese subjects.

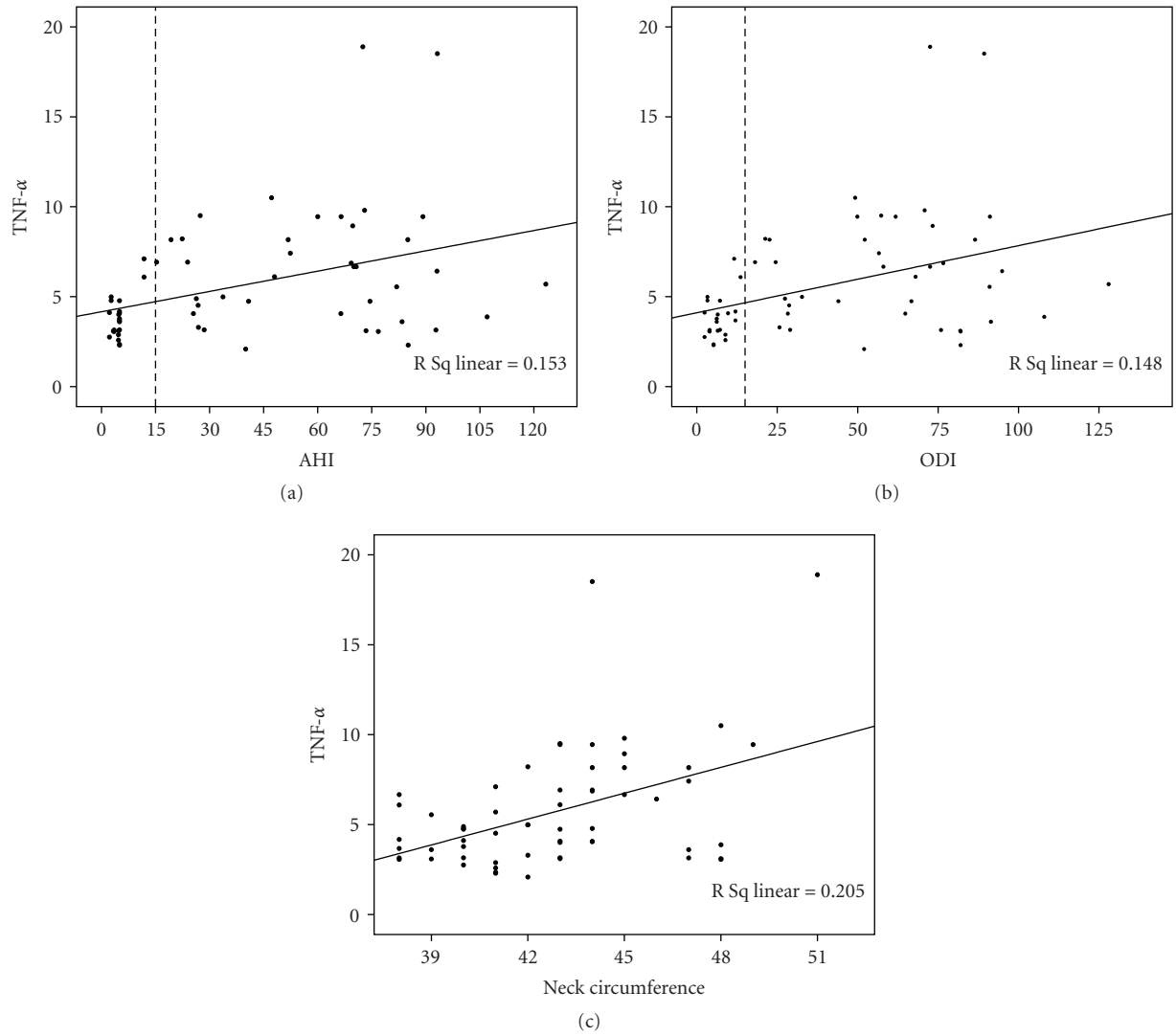

Figure 1: Association between TNF-α levels and AHI (a), ODI (b), and neck circumference (c) in the study population.

By contrast, there was no difference between our two groups in the other three inflammatory markers, that is, CRP, IL-6, and fibrinogen. Prior research has failed to reach a definitive conclusion regarding levels of CRP or IL-6 in OSAS patients. Some authors have reported elevated serum CRP levels [21–24] while others have come to the opposite conclusion [25–28]. Similarly, levels of IL-6, the cytokine inducing the production of CRP, were found elevated in OSAS patients in numerous studies [15, 20, 24], but most of them were criticized for the small number of patient series, the lack of proper match between patients and controls in BMI, and the inclusion of patients with cardiovascular or metabolic disorders [29]. On the contrary, other works did not manage to establish a correlation between OSAS and IL-6 [17, 30]. Interestingly, continuous positive airway pressure therapy (CPAP) has been shown to reduce CRP in patients with adequate compliance to treatment [31]. It appears, therefore, that the role of CRP and IL-6 in OSAS is far from settled and that additional research is required to shed more light on this issue. In this endeavor for further elucidation of the role of CRP and IL-6 in OSAS, our report adds that these inflammatory markers are not increased in obese OSAS subjects as compared to their non-apneic peers.

Fibrinogen levels are increased in OSAS patients, according to previous workers [32–34], indicating a predisposition for coagulation and atherosclerosis. Conversely, the present study failed to demonstrate a difference in fibrinogen levels between our two groups of obese individuals, suggesting that the presence of OSAS probably does not play an important role in the upregulation of fibrinogen levels. Again, the discrepancy between our results and those of previous research may be, partly at least, explained by the different setting (patients from obesity clinic versus those from a sleep unit). Nonetheless, further large-scale studies to re-examine fibrinogen levels in OSAS would be appreciated.

TNF-α levels showed a significant positive correlation with AHI and ODI. The latter corroborates the finding by Ryan et al. [17] where ODI was the strongest predictor of

TNF-α levels, indicating the role of intermittent hypoxia and re-oxygenation in the pathogenesis of inflammation through the activation of NF-κB, a transcription factor that has an important role in inflammatory responses [35]. An additional finding was the correlation between CRP levels and indices of nocturnal hypoxia, like average or minimum SpO_2. Taken together, these results suggest that hypoxia, manifested as repetition of desaturations or as lower levels of average or minimum SpO_2, is probably the major contributor in the activation of inflammation in OSAS.

Moreover, a significantly greater neck circumference was noted in OSAS patients, despite the lack of difference in other characteristics of obesity, like BMI or WHR. This observation is common in OSAS patients and many authors have emphasized the association between neck circumference and apneas [36]. Additionally, a positive association between TNF-α levels and neck circumference was observed in our study population while we did not manage to establish a correlation between this marker and other indices of obesity.

The limitations of this study may be outlined as follows. First, the majority of recruited subjects were male. Therefore, some reservation is needed in applying our findings to females. Additionally, although waist circumference is increasingly recognized to be the best indicator for the degree of visceral adiposity, we did not employ MRI to measure directly fat amount and fat distribution, as this would substantially increase the cost of the study [37, 38].

The present work may have the following practical implications. Among obese subjects, those with new diagnosis of OSAS exhibit more pronounced inflammation, as evidenced by the increased levels of TNF-α. Moreover, it is worth noting that application of CPAP has already been shown to reduce levels of this cytokine in overt OSAS [39]. Hence, it is conceivable that inflammation, in general, and TNF-α, in particular, may prove a useful target for therapeutic intervention in obese patients. Vice versa, given that repeated episodes of hypoxia were identified as a major contributor in the activation of inflammation among newly diagnosed OSAS patients in this study, one could also argue that early evaluation of the obese patient for the presence of OSAS might be anticipated to help towards mitigation of chronic inflammation. Certainly, the interaction between inflammation and OSAS in obese patients is complicated and merits further study.

5. Conclusions

The present study shows that, among obese subjects, TNF-α levels are increased in the presence of OSAS, as compared to non-apneic BMI-matched controls. Additionally, TNF-α levels are associated with respiratory disturbance during sleep, as depicted by AHI and ODI as well as larger neck circumference, a common feature of OSAS patients. Our results suggest a role for OSAS in promoting inflammation among obese subjects, possibly mediated by TNF-α. Further work is now awaited to confirm these findings in larger series, as well as to investigate the potential role of early therapeutic intervention in the obesity clinic to alleviate chronic inflammation.

Abbreviations

BMI: Body Mass Index
WHR: Waist to Hip ratio
BP: Blood pressure
FEV_1: Forced Expiratory Volume in 1st second
FVC: Forced Vital Capacity
ESS: Epworth Sleepiness Scale
AHI: Apnea Hypopnea Index
ODI: Oxygen Desaturation Index
$avSpO_2$: Average saturation during sleep (in pulse oxymetry)
$minSpO_2$: Minimum saturation during sleep (in pulse oxymetry)
$t < 90$: Sleep time with SpO_2 <90%
TST: Total sleep time.

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Research Article

Leptin Administration Downregulates the Increased Expression Levels of Genes Related to Oxidative Stress and Inflammation in the Skeletal Muscle of *ob/ob* Mice

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Obese leptin-deficient *ob/ob* mice exhibit a low-grade chronic inflammation together with a low muscle mass. Our aim was to analyze the changes in muscle expression levels of genes related to oxidative stress and inflammatory responses in leptin deficiency and to identify the effect of *in vivo* leptin administration. *Ob/ob* mice were divided in three groups as follows: control *ob/ob*, leptin-treated *ob/ob* (1 mg/kg/d) and leptin pair-fed *ob/ob* mice. Gastrocnemius weight was lower in control *ob/ob* than in wild type mice ($P < .01$) exhibiting an increase after leptin treatment compared to control and pair-fed ($P < .01$) *ob/ob* animals. Thiobarbituric acid reactive substances, markers of oxidative stress, were higher in serum ($P < .01$) and gastrocnemius ($P = .05$) of control *ob/ob* than in wild type mice and were significantly decreased ($P < .01$) by leptin treatment. Leptin deficiency altered the expression of 1,546 genes, while leptin treatment modified the regulation of 1,127 genes with 86 of them being involved in oxidative stress, immune defense and inflammatory response. Leptin administration decreased the high expression of *Crybb1, Hspb3, Hspb7, Mt4, Cat, Rbm9, Serpinc1* and *Serpinb1a* observed in control *ob/ob* mice, indicating that it improves inflammation and muscle loss.

1. Introduction

Obesity is associated with a low-grade proinflammatory state resulting in an increase of circulating cytokines and inflammatory markers [1]. Inflammatory cytokines have been involved in the impairment of insulin signaling, thus providing molecular links between inflammation and insulin resistance [2]. Inflammation reportedly produces metabolic alterations in skeletal muscle with both inflammatory response and insulin resistance being associated with loss of muscle mass by decreased protein synthesis and increased proteolysis [3–5]. Recently, our group has shown that leptin reverses muscle loss of *ob/ob* mice by inhibiting the activity of the transcriptional factor forkhead box class O3a (FoxO3a) [6].

Leptin is an adipocyte-derived peptidic hormone [7] that inhibits food intake and increases thermogenesis by acting through its hypothalamic receptors [8, 9]. Leptin-deficient *ob/ob* mice are obese, hyperphagic, exhibit type 2 diabetes, decreased body temperature and hypogonadotropic hypogonadism [10]. Leptin is a member of the long-chain helical cytokine family and its receptors, which belong to the class I cytokine receptors, are present in bone marrow and spleen as well as on peripheral monocytes and lymphocytes [1]. Leptin increases in response to acute infection and sepsis and it has been reported to exert a profound influence on the function and proliferation of T lymphocytes and natural killer cells [11], on the phagocytosis of macrophages/monocytes [12], and to have a direct effect on the secretion of anti- and proinflammatory cytokines [13]. In this regard, impaired cellular and humoral immunity have been shown in leptin-deficient *ob/ob* mice as well as in leptin receptor-deficient *db/db* mice [14, 15]. These studies reflect the molecular nature of leptin as a cytokine and are consistent with leptin signaling playing a pivotal role in the pathogenesis of obesity-associated inflammation and muscle loss.

In the present paper, gastrocnemius muscle samples from wild type and *ob/ob* mice were analyzed for mRNA presence of over 41,000 transcripts by microarray analysis to identify genes involved in inflammation and oxidative stress that are affected by leptin deficiency and leptin administration in *ob/ob* mice. It was shown that leptin increases the gastrocnemius weight and reduces the high expression levels of genes related to the obesity-associated low-grade inflammation in skeletal muscle of *ob/ob* mice.

2. Material and Methods

2.1. Animals and Treatments. Ten-week-old male genetically obese *ob/ob* mice (C57BL/6J) ($n = 15$) and their lean control littermates wild type ($n = 5$) supplied by Harlan (Barcelona, Spain) were housed in a room with controlled temperature ($22 \pm 2^{\circ}$C) and a 12:12 light-dark cycle (lights on at 08:00 am). Body weight of *ob/ob* mice was measured before randomization into control, leptin-treated (1 mg/kg/d) and pair-fed groups ($n = 5$ per group). The control and pair-fed groups received vehicle (PBS), while leptin-treated mice were intraperitoneally administered with leptin (Bachem, Bubendorf, Switzerland) twice daily at 08:00 am and 08:00 pm for 28 days. Control and leptin-treated groups were provided with water and food *ad libitum* with a standard rodent chow (2014S Teklad, Harlan), while daily food intake of the pair-fed group was matched to the amount consumed by the leptin-treated group the day before in order to discriminate the inhibitory effect of leptin on appetite. Animals were sacrificed on the 28th day of treatment by CO_2 inhalation 20 hours after the last PBS or leptin administration (in order to avoid picking up effects reflecting an acute response) and after 8 hours of fasting. Serum samples and gastrocnemius muscles were obtained and stored at -80°C. All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609) and were approved by the Ethical Committee for Animal Experimentation of the University of Navarra (080/05).

2.2. Blood Analysis. Serum glucose was analyzed using a sensitive-automatic glucose sensor (Ascensia Elite, Bayer, Barcelona, Spain). Free fatty acid (FFA) concentrations were measured by a colorimetric determination using the NEFA C kit (WAKO Chemicals, Neuss, Germany). Serum glycerol concentrations were evaluated by enzymatic methods as previously described [6]. Serum triglycerides (TG) concentrations were spectrophotometrically determined using a commercial kit (Infinity, Thermo Electron, Melbourne, Australia). Insulin and leptin were determined using specific mouse ELISA kits (Crystal Chem Inc., Chicago, IL, USA). Intra- and interassay coefficients of variation for measurements of insulin and leptin were 3.5% and 6.3%, respectively, for the former, and 2.8% and 5.8%, for the latter. Adiponectin concentrations were also assessed using a mouse ELISA kit (BioVendor Laboratory Medicine, Inc., Modrice, Czech Republic). Intra- and interassay coefficients of variation for adiponectin were 2.6% and 5.3%, respectively. Insulin resistance was calculated using the homeostasis model assessment score (HOMA; fasting insulin (μU/mL) $\times$ fasting glucose (mmol/L)/22.5) [16]. An indirect measure of insulin sensitivity was calculated by using the quantitative insulin sensitivity check index (QUICKI; 1/[log(fasting insulin mU/mL) + log(fasting glucose mg/dL)] [17].

Lipid peroxidation was analyzed by the measurement of thiobarbituric acid reactive substances (TBARS) in serum and gastrocnemius as previously described by Conti et al. [18] with some modifications. Since the best-known specific TBARS is malondialdehyde (MDA), we used serum MDA levels, a secondary product of lipid peroxidation, as an indicator of lipid peroxidation and oxidative stress. Gastrocnemius samples (20–30 mg) were homogenized in 20 volumes of phosphate buffer pH 7.4. Serum, muscle homogenates (5 μL) or standard (MDA) were mixed with 120 μL of diethyl thiobarbituric acid (DETBA) 10 mM and vortexed for 5 seconds. The reaction mixture was then incubated at 95°C for 60 minutes. After cooling to room temperature DETBA-MDA adducts were extracted in 360 μL n-butanol vortexing for 1 minute and centrifuged at 1,600 g for 10 minutes at room temperature. Then, the chromophore of the DETBA-MDA adduct was quantified in 200 μL of the upper butanol phase by fluorescence emission at 535 nm with an excitation at 590 nm. MDA equivalents (TBARS) were quantified using a calibration curve prepared using MDA standard working solutions and expressed as serum MDA μM and gastrocnemius MDA μM/mg protein. Protein concentrations were determined using a Bradford protein assay kit (BioRad, Hercules, CA, USA).

2.3. Microarray Experiments and Analysis. Total RNA was extracted from 20–30 mg of gastrocnemius muscle samples by homogenization with an ULTRA-TURRAX T 25 basic (IKA Werke GmbH, Staufen, Germany) using TRIzol reagent (Invitrogen, Barcelona, Spain). RNA was purified using the RNeasy Mini kit (Qiagen, Barcelona, Spain) and treated with DNase I (RNase-free DNase Set, Qiagen) in order to remove any trace of genomic DNA.

Gene expression analyses were conducted using the Agilent Whole Mouse Genome array (G4121B, Agilent Technologies, Santa Clara, CA, USA) containing ~41,000 mouse genes and transcripts. Fluorescence-labeled cDNA probes were prepared from 1 μg of total RNA from each sample (5 animals per group) to be subsequently amino-allyl labeled and amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX, USA). Aliquots (1.2 μg) of amplified aRNA were fluorescently labeled using Cy3/Cy5 (Amersham Biosciences, Buckinghamshire, UK) and then appropriately combined and hybridized to Agilent microarrays. Hybridizations were performed following a reference design, where control samples were pools of RNA from all individual samples. Two hybridizations with fluor reversal (Dye-swap) were performed for each sample. After washing, microarray slides were scanned using a Gene Pix 4100A scanner (Axon Instruments, Union City, CA, USA) and image quantization was performed using the software GenePiX Pro 6.0. Gene expression data for all replicate experiments were analyzed using the GeneSpring GX software version 7.3.1 (Agilent

Table 1: Sequences of the primers and Taqman probes used in the Real-Time PCR.

Gene	Gene Symbol	GenBank accesión number	Oligonucleotide sequence (5′-3′)
Peroxisome proliferator-activated receptor-γ coactivator-1α	*Pgc1a*	NM_008904	Forward: GTCTGAAAGGGCCAAACAGAGA
			Reverse: TCAATTCTGTCCGCGTTGTG
			Probe: FAM-AGCAGAAAGCAATTGAAGAGCGCCGT-TAMRA
Forkhead box O1	*Foxo1*	NM_019739	Forward: GCGGGCTGGAAGAATTCAAT
			Reverse: TCCTTCATTCTGCACTCGAATAAACT
			Probe: FAM-CGCCACAATCTGTCCCTTCACA-TAMRA
Muscle atrophy F box	*MAFbx*	NM_026346	Forward: CCATCCTGGATTCCAGAAGATTC
			Reverse: TCAGGGATGTGAGCTGTGACTTT
			Probe: FAM-CTACGTAGTAAGGCTGTTGGAGCTGAT-TAMRA
Muscle RING finger 1	*MuRF1*	NM_001039048	Forward: CGCCATGAAGTGATCATGGA
			Reverse: TCCTTGGAAGATGCTTTGCA
			Probe: FAM-TGTACGGCCTGCAGAGGAACCTGAAA-TAMRA

Technologies). Clustering was accomplished with the Gene and Condition Tree algorithms. In addition, Gene Ontology database (http://babelomics.bioinfo.cipf.es) and the KEGG website (http://www.genome.ad.jp/kegg/pathway) were used in conjunction with GeneSpring (http://www.agilent.com/ch-em/genespring) to identify pathways and functional groups of genes. All microarray data reported are described in accordance with MIAME guidelines (http://www.mged.org/Workgroups/MIAME/miame.html). More information regarding the microarray experiments can be found at the EMBL-European Bioinformatics Institute (http://www.ebi.ac.uk/aerep/login. ArrayExpress accession number: E-MEXP-1831). To validate the microarray data, a number of representative differentially expressed genes were selected to be individually studied by Real-Time PCR (7300 Real Time PCR System, Applied Biosystems, Foster City, CA, USA) ($n = 5$ per group) as previously described [19]. Primers and probes were designed using the software Primer Express 2.0 (Applied Biosystems) and purchased from Genosys (Sigma, Madrid, Spain) (Table 1).

2.4. Statistical Analysis. Data are expressed as mean ± standard error of the mean (SEM). Differences between groups were assessed by Kruskal-Wallis followed by Mann Whitney's U test. As previously outlined, Gene Ontology groupings were used to identify pathways significantly affected by leptin deficiency as opposed to its administration. Furthermore, statistical comparisons for microarray data to identify differentially expressed genes across different groups were performed using one-way ANOVA and Student's t-tests as appropriate. Spearman's correlations were used to evaluate the relations among different variables. All statistical analyses were performed by using the SPSS statistical program version 15.0 for Windows (SPSS, Chicago, IL, USA) and statistical significance was defined as $P < .05$.

3. Results

3.1. Leptin Treatment Improves the Metabolic Profile of ob/ob Mice. The morphological and biochemical characteristics of wild type and *ob/ob* mice are reported in Table 2. As expected, leptin treatment corrected the obese and diabetic phenotype of *ob/ob* mice. Body weight was significantly higher ($P < .01$) in the control *ob/ob* group as compared to wild type mice. Leptin-treated mice exhibited a decreased body weight ($P < .01$) as compared to control and pair-fed *ob/ob* animals. Importantly, leptin treatment normalized body weight of *ob/ob* mice as compared to wild type ($P = .690$). In addition, the gastrocnemius of control *ob/ob* mice exhibited a lower ($P < .01$) muscle weight than that of wild type mice and it was increased ($P < .01$) by leptin administration in comparison with that of control and pair-fed *ob/ob* rodents. As depicted in Table 2, higher fasting glucose ($P < .05$) and insulin ($P < .01$) concentrations were observed in the control *ob/ob* mice compared to wild types. Although no differences in glucose concentrations were observed in pair-fed as compared to leptin-treated *ob/ob* mice, higher serum insulin concentrations ($P < .05$) were detected in the pair-fed animals than in the leptin-treated *ob/ob* group. Furthermore, leptin administration normalized both the glucose and insulin levels in *ob/ob* mice compared to wild types. These data suggest that leptin increases the insulin sensitivity in peripheral tissues, as evidenced by the lower HOMA and higher QUICKI indices ($P < .01$) in the leptin-treated in comparison with the control *ob/ob* animals. Serum glycerol was markedly increased ($P < .05$) in the control *ob/ob* mice, while FFA and TG levels remained unchanged

Table 2: Total body and skeletal muscle weights and biochemical characteristics of wild type and *ob/ob* mice.

	wild type	control *ob/ob*	pair-fed *ob/ob*	leptin-treated *ob/ob*
Body weight (g)	25.6 ± 0.3	47.8 ± 4.9[b]	35.7 ± 0.7	24.7 ± 1.2[d,f]
Gastrocnemius (mg)	142.9 ± 3.4	90.7 ± 10.0[b]	68.5 ± 1.6	104.9 ± 2.6[b,f]
Gastrocnemius (mg/g)	5.59 ± 0.12	1.91 ± 0.11[b]	1.92 ± 0.07	4.28 ± 0.15[b,d,f]
Glucose (mg/dL)	149 ± 42	430 ± 59[a]	160 ± 24[d]	178 ± 29[d]
FFA (mmol/L)	1.62 ± 0.49	1.61 ± 0.30	1.65 ± 0.12	0.78 ± 0.13[c,f]
Glycerol (mmol/L)	42.8 ± 6.7	81.6 ± 19.6[a]	39.6 ± 4.9[c]	12.3 ± 4.7[a,d,f]
TG (mg/dL)	122 ± 18	169 ± 32	151 ± 10	86 ± 17[e]
Insulin (ng/mL)	0.42 ± 0.09	8.60 ± 1.51[b]	2.40 ± 0.68[c]	0.47 ± 0.09[d,e]
Adiponectin (μg/mL)	30.2 ± 3.0	28.3 ± 5.4	39.1 ± 1.8	40.2 ± 3.0
Leptin (ng/mL)	1.36 ± 0.42	UD	UD	3.48 ± 1.02
HOMA	4.3 ± 1.8	202.4 ± 33.8[b]	25.8 ± 10.4[d]	5.12 ± 1.1[d]
QUICKI	0.333 ± 0.023	0.205 ± 0.003[b]	0.263 ± 0.015[d]	0.311 ± 0.016[d]

Data are mean ± SEM ($n = 5$ per group). Differences between groups were analyzed by Kruskal-Wallis followed by Mann Whitney's U test. [a]$P < .05$ and [b]$P < .01$ *versus* wild type. [c]$P < .05$ and [d]$P < .01$ *versus ob/ob*. [e]$P < .05$ and [f]$P < .01$ *versus* pair-fed *ob/ob*. FFA: free fatty acids. TG: triglycerides. UD: undetectable. HOMA: homeostasis model assessment. QUICKI: quantitative insulin sensitivity check index.

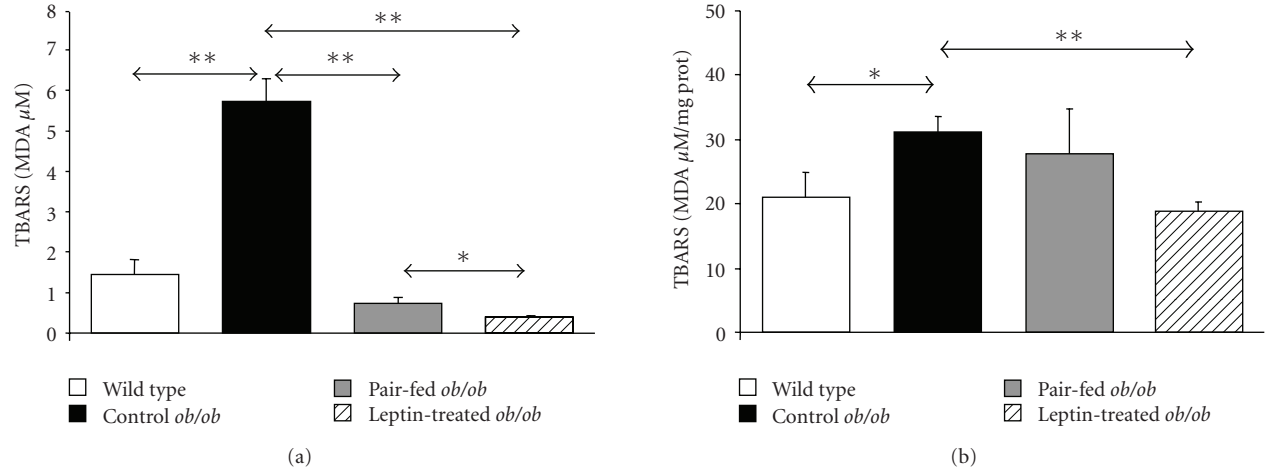

Figure 1: Leptin reduces TBARS concentrations in *ob/ob* mice. Thiobarbituric acid reactive substances (TBARS) presented as concentrations of malondialdehyde (MDA μM) in serum (a) and gastrocnemius muscle (MDA μM/mg prot) (b) of wild type (open), control *ob/ob* (closed), pair-fed *ob/ob* (gray) and leptin-treated *ob/ob* (striped) mice ($n = 5$ per group). Data are expressed as mean ± SEM. *$P < .05$ and **$P < .01$ by Kruskal-Wallis followed by Mann Whitney's U test.

as compared to wild type mice. Interestingly, leptin not only decreased circulating concentrations of FFA ($P < .05$) and glycerol ($P < .01$) levels as compared to control *ob/ob* mice, but also FFA ($P < .01$), glycerol ($P < .01$) and TG ($P < .05$) concentrations as compared to pair-fed mice. Leptin administration to *ob/ob* mice reduced serum glycerol concentrations ($P = .032$) and tended to decrease FFA ($P = .095$) as compared to wild types. Furthermore, leptin treatment increased the low concentrations of adiponectin of *ob/ob* mice, but the differences fell out of statistical significance ($P = .095$).

Control *ob/ob* mice exhibited significantly higher serum TBARS than wild type littermates ($P < .01$), which were significantly reduced after leptin administration as compared to the control ($P < .01$) and pair-fed ($P < .05$) *ob/ob* groups (Figure 1(a)). In addition, leptin decreased ($P < .01$) the high concentrations of MDA measured in the gastrocnemius muscle of control *ob/ob* mice, while this effect was not observed in the pair-fed group (Figure 1(b)). Serum and gastrocnemius TBARS levels were positively associated with body weight, FFA, insulin, and the HOMA index. Oppositely, TBARS levels were negatively associated with adiponectin and the QUICKI index both in serum and muscle. Importantly, a high positive relation were found between serum and gastrocnemius concentrations of TBARS ($\rho = 0.63$, $P = .003$) (Table 3).

3.2. Leptin Induces Changes in Gene Expression—Effect of Leptin on Genes Invoved in Oxidative Stress and Inflammation. Differential gene expression profiles in gastrocnemius muscle of wild type and *ob/ob* groups were compared by microarray analysis. Only genes whose mRNA levels were changed

Table 3: Bivariate analysis of the correlations between TBARS concentrations in serum and the gastrocnemius muscle with anthropometric and biochemical variables in wild type and *ob/ob* mice.

	Serum TBARS		Gastrocnemius TBARS	
	ρ	P	ρ	P
Body weight	0.57	.009	0.46	.040
Glucose	0.44	.055	0.38	.103
FFA	0.54	.015	0.59	.007
Glycerol	0.49	<.001	0.44	.053
TG	0.44	.054	0.44	.050
Insulin	0.49	.027	0.52	.020
Adiponectin	−0.51	.022	−0.53	.016
QUICKI	−0.48	.031	−0.48	.033
HOMA	0.53	.019	0.51	.025

Values are Spearman's correlation coefficients (ρ) and associated P values. TBARS: thiobarbituric acid reactive substances. FFA: free fatty acids. TG: triglycerides. HOMA: homeostasis model assessment. QUICKI: quantitative insulin sensitivity check index.

1.5-fold or higher and identified as significantly changed by statistical analysis were designated as differentially expressed genes. Applying these criteria, microarray data showed that 7,582 genes were differentially expressed by leptin deficiency and leptin administration in *ob/ob* mice. In particular, leptin deficiency altered the expression of 1,127 genes between wild type and control *ob/ob* mice. Of these, 580 were upregulated and 547 were downregulated in *ob/ob* mice. Leptin treatment modified the expression of 1,546 genes in *ob/ob* mice, upregulating 512 and repressing 1,034. In addition, leptin repressed 736 genes that were upregulated in gastrocnemius muscle of control *ob/ob* and increased the transcript levels of 846 downregulated genes. Functional enrichment analysis using GeneOntology and KEGG databases revealed that the set of genes with altered expression levels induced by leptin deficiency and administration represents a broad spectrum of biological processes. However, for the purpose of the present paper we focused on the effects of leptin on the set of genes encoding proteins involved in oxidative stress and inflammation. Table 4 shows that leptin deficiency and leptin administration altered the expression of a large number of genes involved in oxidative stress and inflammation. The biological processes mainly affected between control *ob/ob* mice and wild types included "response to oxidative stress" ($P = .0006$), "response to stress" ($P = .0031$) and "acute-phase response" ($P = .023$). Furthermore, several processes regulating proliferation, differentiation, and activity of lymphocytes were also significantly affected by leptin deficiency. Importantly, comparison of leptin-treated and control *ob/ob* groups showed that leptin administration altered the expression of genes implicated in the "positive regulation of lymphocyte activation" ($P = .0003$), "positive regulation of immune response" ($P = .0032$) and "response to stress" ($P = .0187$), as well as genes involved in the "chaperone cofactor dependent protein folding" ($P = .0023$).

Noteworthy, leptin reduced the expression of several genes related to inflammatory conditions. DNA microarray analysis showed that 86 genes encoding proteins related to defense, stress, and inflammatory responses were altered in the gastrocnemius muscle of control *ob/ob* mice and modified by leptin administration. Leptin reduced the mRNA levels of various isoforms of the family of heat shock proteins (HSPs) (*Dnajc16, Dnaja4*, *Dnajb4*, *Hspa2*, *Hspa4*, and *Hspb7*), metallothioneins (*Mt2*, *Mt4*), crystallins (*Cryab*, *Crybb1*) and RNA binding proteins (RBMs) (*Rbm9*, *Rbm22*) in *ob/ob* mice (Table 5). In addition, histocompatibility 2, complement component factor B *H2-Bf* and several genes of the acute-phase response or inflammatory processes, such as kallikrein 5 (*Klk5*), and serine (or cysteine) proteinase inhibitor clade C member 1 (*Serpinc1)* and clade B member 1a (*Serpinb1a*), displayed an increased expression in *ob/ob* mice that was reduced by leptin administration. On the contrary, gene expression of *Cryl1*, *Hsp105*, *Rbm5,* and *H2-Aa* were enhanced in *ob/ob* mice after treated with leptin. Pair-feeding, which accounts for the decrease in food intake that is independent of the direct action of leptin, altered the expression of 1,960 genes, upregulating 984 while downregulating 976 genes. In the context of a food intake reduction as compared to the simple effect due to the caloric restriction, leptin administration further significantly altered the expression of genes involved in processes encompassing "immune response" ($P = 5.53e^{-8}$) "defense response" ($P = 3.83e^{-6}$), "response to oxidative stress" ($P = 2.99e^{-5}$), "positive regulation of T cell activation" ($P = .0003$) and "positive regulation of immune cell mediated cytotoxicity" ($P = .0004$) (Table 4). In particular, the gene array analysis provided evidence for elevated *Hspa4*, *Mt4*, *Crybb1,* and *Serpinb8* mRNA levels in the pair-fed group as compared to the leptin-treated *ob/ob* mice (Table 6). On the contrary, leptin increased the gene expression of *H2-Ab1* and *H2-Eb1* in *ob/ob* mice. To confirm the microarray data, the mRNA expression of several representative transcripts was analyzed by Real-Time PCR (Figure 2). In this sense, leptin administration reduced the mRNA levels of the muscle atrophy-related transcription factor forkhead box O1 (*Foxo1*) and of the E3 ubiquitin-ligases muscle atrophy F-box (*MAFbx*) and muscle RING finger 1 (*MuRF1*) in leptin-treated *ob/ob* mice, while no effect of leptin was evidenced on the mRNA levels of the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (*Pgc1 α*). The expression of the selected genes was concordant with that of the microarray.

4. Discussion

Obesity is accompanied by a chronic proinflammatory state associated not only with insulin resistance, but also with muscular atrophy [4, 5]. Our study provides evidence that leptin constitutes a negative regulator of oxidative stress and inflammation in the gastrocnemius, which is a representative skeletal muscle of the whole skeletal musculature. This statement is supported by findings reported herein: (a) leptin deficiency is accompanied by systemic and skeletal muscle oxidative stress, muscle inflammation, and reduced muscle mass; (b) systemic and skeletal muscle oxidative stress, muscle atrophy and inflammation of *ob/ob* mice are reversed

Table 4: Biological processes according to Gene Ontology (GO) and number of genes altered by leptin deficiency, leptin administration, and pair-feeding in the gastrocnemius muscle of wild type and *ob/ob* mice.

Category	Genes in Category	wild type *vs ob/ob*		*ob/ob vs* leptin		leptin *vs* pair-fed	
		Altered genes	*P* value	Altered genes	*P* value	Altered genes	*P* value
GO:6950: response to stress	**1156**	**61**	**.00314**	**69**	**.0187**	**22**	.0757
GO:6952: defense response	**1010**	**43**	.182	**47**	.510	**33**	**3.83e^{-6}**
GO:6955: immune response	**835**	**36**	.186	**45**	.165	**33**	**5.53e^{-8}**
GO:45321: immune cell activation	**230**	**9**	.475	**13**	.270	**6**	.0974
GO:46649: lymphocyte activation	**208**	**9**	.359	**13**	.170	**6**	.0673
GO:6954: inflammatory response	**199**	**4**	.938	**4**	.984	**2**	.7590
GO:50776: regulation of immune response	**148**	**9**	.097	**12**	**.0426**	**8**	**.00102**
GO:6959: humoral immune response	**123**	**7**	.169	**8**	.211	**4**	.0891
GO:42110: T cell activation	**112**	**5**	.396	**7**	.263	**5**	**.0191**
GO:30098: lymphocyte differentiation	**107**	**8**	**.0441**	**8**	.123	**4**	.0597
GO:42113: B cell activation	**101**	**3**	.724	**7**	.188	**3**	.1610
GO:6800: oxygen and reactive oxygen species metabolism	**92**	**11**	**.00056**	**7**	.135	**7**	**.00027**
GO:50778: positive regulation of immune response	**91**	**7**	.0508	**11**	**.0032**	**8**	**3.6e^{-5}**
GO:51249: regulation of lymphocyte activation	**89**	**7**	**.046**	**10**	**.00808**	**5**	**.0076**
GO:19882: antigen presentation	**81**	**9**	**.0029**	**9**	**.0125**	**8**	**1.53e^{-5}**
GO:31098: stress-activated protein kinase signaling pathway	**80**	**8**	**.00921**	**5**	.313	**1**	.6690
GO:30333: antigen processing	**78**	**11**	**.00013**	**13**	**5.65e^{-5}**	**8**	**1.16e^{-5}**
GO:7254: JNK cascade	**75**	**8**	**.00629**	**4**	.461	**1**	.6450
GO:46651: lymphocyte proliferation	**67**	**2**	.712	**5**	.199	**2**	.2340
GO:6979: response to oxidative stress	**65**	**9**	**.0006**	**7**	**.0303**	**7**	**2.99e^{-5}**
GO:50863: regulation of T cell activation	**62**	**5**	.0779	**6**	.0667	**5**	**.0016**
GO:7249: I-kappaB kinase/NF-kappaB cascade	**61**	**2**	.663	**3**	.542	**3**	.0512
GO:51251: positive regulation of lymphocyte activation	**58**	**6**	**.0196**	**10**	**.0003**	**5**	**.00118**
GO:30217: T cell differentiation	**54**	**5**	**.0481**	**6**	**.0380**	**4**	**.00638**
GO:9266: response to temperature stimulus	**54**	**12**	**4.78e^{-7}**	**13**	**7.96e^{-7}**	**1**	.5260
GO:30183: B cell differentiation	**50**	**2**	.554	**3**	.410	**2**	.1500
GO:50670: regulation of lymphocyte proliferation	**46**	**2**	.509	**3**	.360	**1**	.4700
GO:50864: regulation of B cell activation	**46**	**2**	.509	**5**	.0606	**2**	.1310
GO:42087: cell-mediated immune response	**44**	**1**	.809	**1**	.876	**2**	.1220

Table 4: Continued.

Category	Genes in Category	wild type *vs ob/ob*		*ob/ob vs* leptin		leptin *vs* pair-fed	
		Altered genes	*P* value	Altered genes	*P* value	Altered genes	*P* value
GO:50777: negative regulation of immune response	**43**	**3**	.210	**2**	.599	**1**	.4480
GO:50870: positive regulation of T cell activation	**43**	**5**	**.0203**	**6**	**.0137**	**5**	**.000294**
GO:42088: T-helper 1 type immune response	**41**	**1**	.786	**1**	.857	**2**	.1080
GO:9408: response to heat	**40**	**9**	**1.17e^{-5}**	**12**	**1.54e^{-7}**	**1**	.4240
GO:45619: regulation of lymphocyte differentiation	**36**	**6**	**.00186**	**5**	**.0242**	**4**	**.00144**
GO:42100: B cell proliferation	**32**	**1**	.699	**5**	**.0150**	**2**	.0709
GO:19884: antigen presentation, exogenous antigen	**31**	**9**	**1.17e^{-6}**	**9**	**7.62e^{-6}**	**8**	**6.81e^{-9}**
GO:50851: antigen receptor-mediated signaling pathway	**30**	**1**	.676	**3**	.160	**1**	.3390
GO:50871: positive regulation of B cell activation	**30**	**1**	.676	**5**	**.0115**	**2**	.0633
GO:51250: negative regulation of lymphocyte activation	**30**	**2**	.304	**1**	.759	**1**	.3390
GO:50671: positive regulation of lymphocyte proliferation	**29**	**2**	.290	**3**	.149	**1**	.3300
GO:1909: immune cell mediated cytotoxicity	**27**	**2**	.262	**2**	.358	**3**	**.00584**
GO:45580: regulation of T cell differentiation	**26**	**5**	**.00232**	**5**	**.00617**	**4**	**.00041**
GO:30888: regulation of B cell proliferation	**24**	**1**	.594	**3**	.0975	**1**	.2820
GO:45621: positive regulation of lymphocyte differentiation	**22**	**4**	**.00788**	**5**	**.00288**	**3**	**.00323**
GO:19886: antigen processing, exogenous antigen via MHC class II	**21**	**9**	**2.37e^{-8}**	**8**	**2.45e^{-6}**	**8**	**1.98e^{-10}**
GO:45058: T cell selection	**20**	**2**	.167	**1**	.613	**3**	**.00244**
GO:50868: negative regulation of T cell activation	**20**	**1**	.528	**1**	.613	**1**	.2410
G O:42591: antigen presentation, exogenous antigen via MHC class II	**19**	**6**	**4.42e^{-5}**	**6**	**.000157**	**6**	**1.47e^{-7}**
GO:45582: positive regulation of T cell differentiation	**19**	**4**	**.00456**	**5**	**.00143**	**3**	**.0021**
GO:1910: regulation of immune cell mediated cytotoxicity	**18**	**2**	.141	**2**	.202	**3**	**.00178**
GO:19724: B cell mediated immunity	**18**	**1**	.491	**1**	.574	**1**	.2200
GO:45577: regulation of B cell differentiation	**16**	**1**	.452	**1**	.532	**2**	**.0198**
GO:46328: regulation of JNK cascade	**16**	**1**	.452	**2**	.168	**1**	.1980
GO:30890: positive regulation of B cell proliferation	**14**	**1**	.409	**3**	**.0246**	**1**	.1760
GO:45060: negative thymic T cell selection	**14**	**1**	.409	**1**	.485	**1**	.1760
GO:51085: chaperone cofactor dependent protein folding	**13**	**2**	.0809	**4**	**.00234**	**3**	**.00066**

Table 4: Continued.

Category	Genes in Category	wild type *vs ob/ob*		*ob/ob vs* leptin		leptin *vs* pair-fed	
		Altered genes	*P* value	Altered genes	*P* value	Altered genes	*P* value
GO:1912: positive regulation of immune cell mediated cytotoxicity	**11**	**1**	.338	**1**	.407	**3**	**.00039**
GO:48002: antigen presentation, peptide antigen	**10**	**5**	**$1.45e^{-5}$**	**5**	**$4.39e^{-5}$**	**4**	**$6.8e^{-6}$**
GO:48005: antigen presentation, exogenous peptide antigen	**7**	**5**	**$1.33e^{-6}$**	**5**	**$4.11e^{-6}$**	**4**	**$1.17e^{-6}$**
GO:45620: negative regulation of lymphocyte differentiation	**6**	**2**	**.0184**	**1**	.248	**1**	.0794
GO:46330: positive regulation of JNK cascade	**4**	**1**	.139	**1**	.173	**1**	.0537
GO:45581: negative regulation of T cell differentiation	**2**	**1**	.0723	**1**	.0905	**1**	**.0272**

P values reflect the significance of change in prevalence of genes in each category under the leptin deficiency (*ob/ob*), leptin administration (leptin) and pair-feeding (pair-fed) conditions in *ob/ob* mice to the expected prevalence of genes in each category. Statistically significant *P* values are highlighted in bold.

by leptin administration independently of the effects of food intake inhibition. Therefore, leptin is able to prevent the muscle atrophy associated with obese and inflammatory states.

Skeletal muscle constitutes an important target for leptin playing a key role on the regulation of lipid and glucose metabolism [20]. Since obese *ob/ob* mice exhibit an increased oxidative stress and impaired immune response [14, 15] and a reduced skeletal muscle mass [21] compared with their lean littermates, we aimed to identify the genes related to inflammatory processes differentially altered by leptin in the gastrocnemius muscle of obese *ob/ob* mice. In particular, 86 transcripts encoding inflammation-related proteins were shown to be modified by exogenous leptin administration. However, it has to be taken into account that many of these genes are multifunctional and may have important functions in other biological processes. Among them, leptin repressed the high expression levels of acute-phase reactants and several members of the HSP and RBM families. In addition, confirming a previous study of our group [6], leptin treatment increased the reduced muscle weight of gastrocnemius muscle of *ob/ob* mice. Taken together, these data suggest that leptin may prevent the obesity-associated inflammatory state and the muscle mass loss related to inflammatory states in leptin-deficient *ob/ob* mice.

Leptin-deficient *ob/ob* and leptin receptor-deficient *db/db* mice display many abnormalities in the immune response similar to those observed in starved animals and malnourished humans [14, 15, 22]. In this respect, exogenous leptin replacement to *ob/ob* mice modulates T cell responses in mice and prevents starvation-induced immunosuppression, suggesting that lack of leptin is directly involved in these immune system abnormalities [23, 24]. In agreement with these studies, our findings show that leptin deficiency and administration differentially regulate biological processes related to the immune response as well as the T and B cell differentiation and activation in gastrocnemius muscle of *ob/ob* mice.

Oxidative stress is defined as the imbalanced redox state in which prooxidants overwhelm the antioxidant capacity, resulting in an increased production of reactive oxygen species (ROS), ultimately leading to oxidative damage of cellular macromolecules. The major ROS is the superoxide anion ($\bullet O_2^-$). Dismutation of $\bullet O_2^-$ by superoxide dismutase (SOD) produces hydrogen peroxide (H_2O_2), a more stable ROS, which, in turn, is converted to water by catalase and glutathione peroxidase (GPx) [25]. Oxidative stress is increased in diabetes [26, 27] with leptin administration reportedly improving insulin sensitivity in normal and diabetic rodents [28–30]. However, the relationship between leptin and oxidative stress has not been clearly exhibited. Leptin stimulates *in vitro* ROS production by inflammatory cells [31] and endothelial cells [32] and the level of systemic oxidative stress in nonobese animals [33, 34], suggesting a "prooxidative" role of leptin. However, administration of recombinant leptin reduces the oxidative stress induced by a high-fat diet in mice [35]. In this sense, findings of our study show a high oxidative stress in diabetic *ob/ob* mice, as reflected by increased TBARS concentrations in serum and the gastrocemius muscle. These observations are in agreement with a large number of studies related to increased plasma TBARS or MDA in diabetic rats [36] and humans [37]. Lipid peroxidation is a common index of free radical mediated injury and induction of antioxidant enzyme is a common cellular response [38]. More importantly, leptin administration decreased serum and gastrocnemius TBARS concentrations as compared to control *ob/ob* mice, with TBARS levels in gastrocnemius muscle from pair-fed *ob/ob* animals remaining very similar to those of control *ob/ob* mice. In this sense, from a molecular perspective, our results further show that transcript levels of *Sod1*, *Gpx3* and glutathione S-transferase π 1 *Gstp1* are downregulated

Table 5: Genes involved in oxidative stress and inflammatory responses altered by leptin in the gastrocnemius muscle of *ob/ob* mice.

GeneBank Number	Gene Symbol	Gene Name	Fold change		Ratio
			ob/ob	leptin	
Genes downregulated by leptin					
NM_009804	*Cat*	*Catalase*	1.47	1.13	0.77
NM_007705	*Cirbp*	*Cold inducible RNA binding protein*	1.68	1.14	0.68
NM_009964	*Cryab*	*Crystallin, α B*	1.32	1.15	0.87
NM_023695	*Crybb1*	*Crystallin, β B1*	2.21	1.39	0.63
NM_023646	*Dnaja3*	*DnaJ (Hsp40) homolog, subfamily A, member 3*	0.95	0.64	0.67
NM_021422	*Dnaja4*	*Heat shock protein, DNAJ-like 4*	0.88	0.30	0.34
NM_018808	*Dnajb1*	*DnaJ (Hsp40) homolog, subfamily B, member 1*	0.44	0.33	0.74
NM_026400	*Dnajb11*	*DnaJ (Hsp40) homolog, subfamily B, member 11*	1.11	0.93	0.84
NM_027287	*Dnajb4*	*DnaJ (Hsp40) homolog, subfamily B, member 4*	1.09	0.60	0.55
NM_019874	*Dnajb5*	*DnaJ (Hsp40) homolog, subfamily B, member 5*	1.03	0.73	0.72
NM_011847	*Dnajb6*	*DnaJ (Hsp40) homolog, subfamily B, member 6 isoform c*	0.70	0.47	0.67
NM_013760	*Dnajb9*	*DnaJ (Hsp40) homolog, subfamily B, member 9*	0.62	0.39	0.63
NM_007869	*Dnajc1*	*DnaJ (Hsp40) homolog, subfamily C, member 1*	0.82	0.52	0.63
NM_028873	*Dnajc14*	*DnaJ (Hsp40) homolog, subfamily C, member 14*	1.12	0.87	0.77
NM_172338	*Dnajc16*	*DnaJ (Hsp40) homolog, subfamily C, member 16*	1.15	0.66	0.57
NM_009584	*Dnajc2*	*DnaJ (Hsp40) homolog, subfamily C, member 2*	1.01	0.82	0.81
NM_008929	*Dnajc3*	*DnaJ (Hsp40) homolog, subfamily C, member 3B*	1.02	0.83	0.82
NM_016775	*Dnajc5*	*DnaJ (Hsp40) homolog, subfamily C, member 5*	0.74	0.50	0.67
NM_010344	*Gsr*	*Glutathione reductase 1*	1.17	0.71	0.61
NM_008180	*Gss*	*Glutathione synthetase*	1.13	0.88	0.78
NM_010357	*Gsta4*	*Glutathione S-transferase, α 4*	1.50	1.46	0.97
NM_010362	*Gsto1*	*Glutathione S-transferase* o *1*	1.42	1.15	0.81
NM_008198	*H2-Bf*	*Histocompatibility 2, complement component factor B*	2.00	1.44	0.72
NM_013558	*Hspa1l*	*Heat shock 70kDa protein 1-like*	1.60	1.04	0.65
NM_008301	*Hspa2*	*Heat shock protein 2*	1.49	0.98	0.65
NM_008300	*Hspa4*	*Heat shock protein 4*	0.92	0.30	0.32
NM_031165	*Hspa8*	*Heat shock protein 8*	0.91	0.57	0.62
NM_010481	*Hspa9a*	*Heat shock protein 9*	1.03	0.88	0.86
NM_024441	*Hspb2*	*Heat shock protein 2*	1.45	1.21	0.83
NM_019960	*Hspb3*	*Heat shock protein 3*	1.66	1.27	0.77
NM_013868	*Hspb7*	*Heat shock protein family, member 7*	1.83	0.35	0.19
NM_008302	*Hspcb*	*Heat shock protein 1, β*	0.86	0.69	0.80
NM_008416	*Junb*	*Jun-B oncogene*	0.59	0.36	0.61
NM_010592	*Jund1*	*Jun D proto-oncogene*	1.49	0.94	0.63
NM_008456	*Klk5*	*Kallikrein 5*	2.23	1.43	0.64
NM_026346	*MAFbx*	*Muscle atrophy F box*	0.65	0.43	0.67
NM_008209	*Mr1*	*Histocompatibility-2 complex class 1-like*	1.19	0.98	0.82
NM_008630	*Mt2*	*Metallothionein 2*	1.11	0.50	0.46
NM_008631	*Mt4*	*Metallothionein 4*	1.27	1.03	0.81
NM_008872	*Plat*	*Plasminogen activator, tissue*	1.56	1.12	0.72
NM_029397	*Rbm12*	*RNA binding motif protein 12*	1.40	1.03	0.74
NM_026453	*Rbm13*	*RNA binding motif protein 13*	1.01	0.87	0.86
NM_026434	*Rbm18*	*RNA binding motif protein 18*	0.94	0.59	0.63
BC080205	*Rbm22*	*RNA binding motif protein 22*	1.14	0.75	0.66
BC040811	*Rbm28*	*Rbm28 protein*	0.69	0.49	0.71
NM_172762	*Rbm34*	*RNA binding motif protein 34*	1.01	0.67	0.66
NM_009032	*Rbm4*	*RNA binding motif protein 4*	1.04	0.81	0.78
NM_148930	*Rbm5*	*RNA binding motif protein 5*	0.69	0.63	0.91
NM_144948	*Rbm7*	*RNA binding motif protein 7*	0.81	0.74	0.91
NM_025875	*Rbm8a*	*RNA binding motif protein 8a*	0.91	0.69	0.76

Table 5: Continued.

GeneBank Number	Gene Symbol	Gene Name	Fold change		Ratio
			ob/ob	leptin	
NM_175387	*Rbm9*	*RNA binding motif protein 9 isoform 2*	1.96	0.46	0.23
NM_025429	*Serpinb1a*	*Serine (or cysteine) proteinase inhibitor, clade B, member 1a*	2.73	2.09	0.77
NM_080844	*Serpinc1*	*Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1*	4.98	1.93	0.39
NM_008871	*Serpine1*	*Serine (or cysteine) proteinase inhibitor, clade E, member 1*	2.12	0.97	0.46
NM_011340	*Serpinf1*	*Serine (or cysteine) proteinase inhibitor, clade F, member 1*	2.43	1.50	0.62
NM_009776	*Serping1*	*Serine (or cysteine) proteinase inhibitor, clade G, member 1*	1.41	1.15	0.81
NM_009776	*Serping1*	*Serine (or cysteine) proteinase inhibitor, clade G, member 1*	1.41	1.15	0.81
NM_013749	*Tnfrsf12a*	*Tumor necrosis factor receptor superfamily, member 12a*	0.78	0.29	0.37
Genes upregulated by leptin					
NM_030004	*Cryl1*	*Crystallin λ 1*	1.25	1.72	1.38
NM_016669	*Crym*	*Crystallin μ*	1.37	1.64	1.19
NM_133679	*Cryzl1*	*Crystallin, ζ (quinone reductase)-like 1*	1.10	1.28	1.16
NM_008161	*Gpx3*	*Glutathione peroxidase 3 isoform 2*	0.47	0.54	1.15
NM_024198	*Gpx7*	*Glutathione peroxidase 7*	1.00	1.34	1.33
NM_010359	*Gstm3*	*Glutathione S-transferase, μ 3*	1.06	1.23	1.17
NM_010360	*Gstm5*	*Glutathione S-transferase, μ 5*	1.09	1.39	1.27
NM_013541	*Gstp1*	*Glutathione S-transferase, π 1*	0.87	1.04	1.20
NM_010361	*Gstt2*	*Glutathione S-transferase, θ 2*	1.21	1.70	1.40
NM_133994	*Gstt3*	*Glutathione S-transferase, θ 3*	1.53	1.69	1.11
NM_010363	*Gstz1*	*Glutathione transferase zeta 1 (maleylacetoacetate isomerase)*	1.13	1.24	1.10
NM_010378	*H2-Aa*	*Histocompatibility 2, class II antigen A, α*	0.46	1.26	2.76
NM_010379	*H2-Ab1*	*Histocompatibility 2, class II antigen A, β1*	0.37	1.04	2.84
NM_010382	*H2-Eb1*	*Histocompatibility 2, class II antigen E β*	0.43	1.03	2.40
NM_010395	*H2-T10*	*Histocompatibility 2, T region locus 10*	1.11	1.41	1.27
NM_013559	*Hsp105*	*Heat shock protein 105*	0.41	0.73	1.79
NM_008303	*Hspe1*	*Heat shock protein 1 (chaperonin 10)*	0.67	0.98	1.48
AK_052911	*MuRF1*	*M muscle RING finger 1*	0.20	0.28	1.43
XM_131139	*Rbm15*	*RNA binding motif protein 15*	0.81	1.34	1.66
NM_197993	*Rbm21*	*RNA binding motif protein 21*	0.67	0.73	1.08
BC029079	*Rbm26*	*Rbm26 protein*	0.75	1.19	1.59
AK087759	*Rbm27*	*RNA binding motif protein 27*	0.88	1.19	1.36
NM_148930	*Rbm5*	*RNA binding motif protein 5*	0.77	1.18	1.55
NM_011251	*Rbm6*	*RNA binding motif protein 6 isoform a*	0.80	0.97	1.21
NM_207105	*Rmcs1*	*histocompatibility 2, class II antigen A, β1*	0.38	0.89	2.37
NM_011454	*Serpinb6b*	*Serine (or cysteine) proteinase inhibitor, clade B, member 6b*	1.06	1.23	1.16
NM_009825	*Serpinh1*	*Serine (or cysteine) proteinase inhibitor, clade H, member 1*	0.65	0.99	1.53
NM_145533	*Smox*	*Spermine oxidase*	0.41	1.23	3.00
AK080908	*Sod1*	*Superoxide dismutase*	0.58	0.62	1.07
NM_011723	*Xdh*	*Xanthine dehydrogenase*	0.68	1.01	1.47

Differential expression of genes is indicated as fold changes with respect to the wild type group presenting only the genes which were significantly different ($P < .05$) between the leptin-treated and the *ob/ob* groups. Ratio: fold change value for leptin-treated between the *ob/ob* groups.

in control *ob/ob* mice as compared to wild type controls being upregulated after leptin treatment. Furthermore, leptin administration also upregulated *Gpx7*, glutathione S-transferase μ 5 (*Gstm5*) and glutathione S-transferase θ 2 (*Gstt2*). On the contrary, the high expression of catalase (*Cat*) was repressed by the exogenous injection of leptin to *ob/ob* mice. These findings are in line with previous observations showing the restoration of the defective antioxidant enzyme activity in plasma of *ob/ob* mice [39] and humans with a leptin gene mutation [40].

Acute-phase reactants have been suggested to contribute to the maintenance of the chronic low-grade inflammation state involved in the progression of obesity and related diseases [41]. Interestingly, our study provides evidence that genes of the acute-phase response were altered in gastrocnemius muscle of *ob/ob* mice, which were counteracted by

Table 6: Genes involved in oxidative stress and inflammatory responses altered by leptin in gastrocnemius muscle of *ob/ob* mice independently of food intake restriction.

GeneBank Number	Gene symbol	Gene name	*Fold change*
Genes downregulated by leptin			
NM_023695	*Crybb1*	*Crystallin, β B1*	0.51
NM_021422	*Dnaja4*	*Heat shock protein, DNAJ-like 4*	0.63
NM_019739	*Foxo1*	*Forkhead box O1*	0.34
NM_008300	*Hspa4*	*Heat shock protein 4*	0.64
NM_013868	*Hspb7*	*Heat shock protein family, member 7*	0.34
NM_010592	*Jund1*	*Jun D proto-oncogene*	0.50
NM_008456	*Klk5*	*Kallikrein 5*	0.46
NM_008491	*Lcn2*	*Lipocalin 2*	0.34
NM_008631	*Mt4*	*Metallothionein 4*	0.63
NM_026346	*MAFbx*	*Muscle atrophy F box*	0.37
AK_052911	*MuRF1*	*M muscle RING finger 1*	0.29
NM_011459	*Serpinb8*	*Serine (or cysteine) proteinase inhibitor, clade B, member 8*	0.38
NM_011459	*Serpinb8*	*Serine (or cysteine) proteinase inhibitor, clade B, member 8*	0.59
NM_008871	*Serpine1*	*Serine (or cysteine) proteinase inhibitor, clade E, member 1*	0.42
Genes upregulated by leptin			
NM_009735	*B2m*	*β-2-microglobulin*	1.92
NM_010361	*Gstt2*	*Glutathione S-transferase, θ 2*	1.94
NM_010379	*H2-Ab1*	*Histocompatibility 2, class II antigen A, β 1*	4.72
NM_010379	*H2-Ab1*	*Histocompatibility 2, class II antigen A, β 1*	3.66
NM_010386	*H2-DMa*	*Histocompatibility 2, class II, locus Dma*	2.35
NM_010387	*H2-DMb1*	*Histocompatibility 2, class II, locus Mb1*	3.31
NM_010382	*H2-Eb1*	*Histocompatibility 2, class II antigen E β*	4.65
NM_013559	*Hsp105*	*Heat shock protein 105*	1,79
AK220167	*Hspa4*	*MKIAA4025 protein*	1,59
NM_207105	*Rmcs1*	*Histocompatibility 2, class II antigen A, β 1*	4.24
NM_207105	*Rmcs1*	*Histocompatibility 2, class II antigen A, β 1*	4.17
NM_009255	*Serpine2*	*Serine (or cysteine) proteinase inhibitor, clade E, member 2*	1.53
NM_009825	*Serpinh1*	*Serine (or cysteine) proteinase inhibitor, clade H, member 1*	2.21
NM_145533	*Smox*	*Spermine oxidase*	4.67

Differential expression of genes is indicated as fold changes presenting only the genes which were significantly different ($P < .05$) between the leptin-treated and the pair-fed *ob/ob* groups.

exogenous leptin administration. Leptin reduced the elevated gene expression of tissue-type plasminogen activator (*Plat*) and lipocalin-2 (*Lcn2*), which are upregulated in many inflammatory conditions [42, 43], including human obesity [44]. In addition, a pivotal role for oxidative stress in the pathogenesis of muscle wasting in disuse and in a variety of pathological conditions is now being widely recognized [45]. A potential link between oxidative stress and muscle atrophy involves the redox regulation of the proteolytic system [46]. Moreover, various inflammatory cytokines induce oxidative stress [47] and muscle atrophy through the activation of the lysosomal [48, 49] and the ubiquitin-proteolysis system [50]. In this context, biological processes related to oxidative stress and inflammatory responses were altered in the gastrocnemius muscle of *ob/ob* mice and improved following leptin treatment. In spite of the usual upregulation of the E3 ubiquitin-ligases MAFbx and MuRF1 in most conditions associated with atrophy, their gene expression levels in *ob/ob* were lower as compared to wild type animals, although no statistically significant differences were observed. Contrarily to what would be expected, leptin administration prevented the increase of both MAFbx and MuRF1 mRNA expression levels induced by pair-feeding in *ob/ob* mice. A plausible explanation for this surprising finding may relate to the fact that in extreme conditions the energy homeostasis system is overriden whereby leptin is able to inhibit muscular protein degradation associated to food intake reduction. These data are in accordance with a previous study of our group evidencing that leptin replacement inhibits the ubiquitin proteolysis system activity in leptin-deficient mice [6]. Muscle atrophy is associated with increased expression of genes coding for RBM proteins which facilitate the translation, protection, and restoration of native RNA conformations during oxidative stress. It has

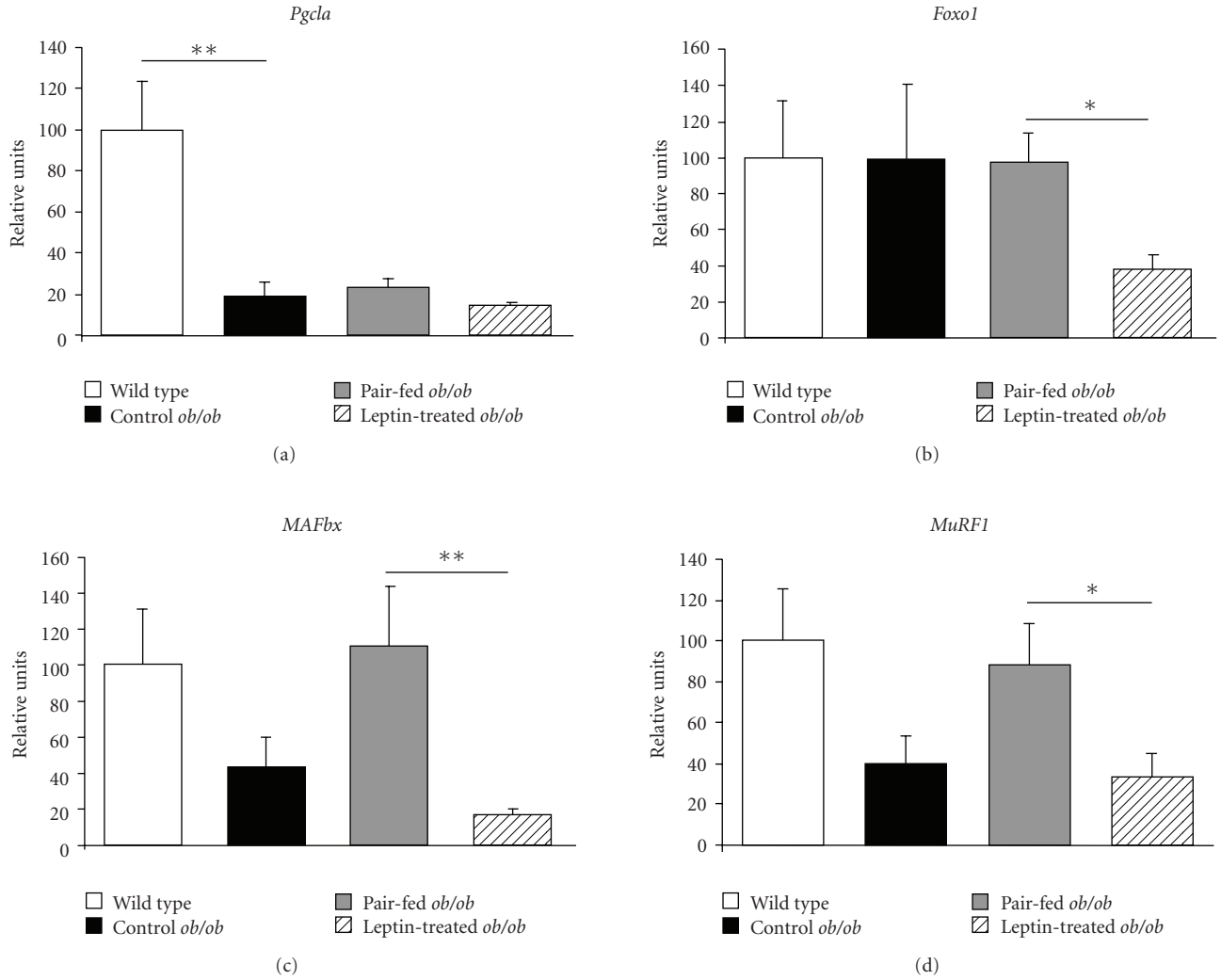

Figure 2: Real-Time PCR analysis of peroxisome proliferator-activated receptor coactivator 1α *(Pgc1a)*, forkhead box class O1 *(Foxo1)*, muscle atrophy F box *(MAFbx)* and muscle RING finger 1 *(MuRF1)* in gastrocnemius muscle of wild type (open), control *ob/ob* (closed), pair-fed *ob/ob* (gray) and leptin-treated *ob/ob* (striped) mice ($n = 5$ per group). Data are presented as mean ± SEM of the ratio between gene expression and 18S rRNA. $^*P < .05$ and $^{**}P < .01$ by Kruskal-Wallis followed by Mann Whitney's U test.

been suggested that the gene expression of RBM proteins may increase as a compensatory mechanism in response to loss of muscle proteins [51, 52]. Other proteins involved in oxidative stress are metallothioneins, endogenous antioxidants [53] that have been shown to be overexpressed in muscle atrophy in rodents [54–56]. In the present work, we have observed that administration of leptin inhibits the gene expression of several members of the RBM (*Rbm9*, *Rbm22*) and metallothioneins (*Mt2*, *Mt4*) families in the gastrocnemius of *ob/ob* mice, suggesting that leptin may modulate the inflammatory and oxidative stress responses and consequently, the muscle loss related to inflammatory states.

Genes involved in the chaperone system were also differentially expressed in *ob/ob* mice as compared to wild types and modified by leptin treatment. HSPs represent a family of molecular chaperones induced in response to cellular stress, responsible for maintaining the structure of proteins and contributing to the repair of damaged or malformed proteins in highly oxidative and lipotoxic conditions. As a result, HSPs are considered antiproteolytic proteins [57]. Muscle atrophy is also associated with an increased gene expression of HSPs [58]. In fact, HSPs are repressed in many rat models of skeletal muscle atrophy [54, 59, 60]. HSP70 is constitutively expressed in skeletal muscle, but its levels are increased in response to oxidative stress [61] with the induction of HSP70 expression by hyperthermia and during inactivity attenuating muscle atrophy [62, 63]. In this regard, a recent study has shown that HSP70 prevents muscle atrophy induced by physical inactivity through inhibition of the muscle atrophy-related transcription factor FoxO3a and the expression of MAFbx and MuRF1 [64]. Among the HSPs, HSP70 and αB-crystallin in particular, are considered negative regulators of muscle cell apoptosis [65, 66] and may inhibit the loss of nuclei taking place during muscle atrophy. In addition, ROS induce the activity of FoxO [67] and gene expression of members of the ubiquitin-proteolysis system

in myotubes [68]. In this sense, our results provide evidence that leptin inhibits the increased gene expression of different members of the HSPs (*Hspb7*, *Dnajc16*, *Hspa4*, *Cryab,* and *Crybb1*) in the gastrocnemius muscle of *ob/ob* mice. Taken together, the elevated expression of HSPs in the control and pair-fed *ob/ob* groups suggests a high defense and stress response in these mice. Moreover, induction of HSPs may confer broader health benefits to patients who are insulin resistant or diabetic [69]. In mammals, caloric restriction has been shown to upregulate HSP induction [70, 71], while expression of HSP72 has been found to be low in skeletal muscle of patients with insulin resistance or type 2 diabetes [72, 73]. Figueiredo et al. [74] have recently shown that leptin downregulates HSP70 gene expression in chicken liver and hypothalamus but not in muscle, which was independent of food intake restriction. On the contrary, Bonior et al. [75] reported an increase in HSP60 gene expression in pancreatic cells by leptin.

Obesity is accompanied by a chronic proinflammatory state resulting in an increase in circulating cytokines and inflammatory markers. In this regard, inflammation produces metabolic alterations in skeletal muscle with both inflammatory response and insulin resistance being associated with muscle mass loss. Findings of our study provide evidence that systemic and skeletal muscle oxidative stress, muscle atrophy and the elevated expression of genes involved in oxidative stress and inflammation of *ob/ob* mice are reversed by leptin administration. Taken together, these data thereby support that leptin is able to prevent the muscle atrophy associated with obese and inflammatory states in *ob/ob* mice. Most obese people develop muscle atrophy in spite of exhibiting high leptin circulating concentrations, which may be explained by the leptin resistance present in these patients. Our paper sheds light on the relation between obesity and the loss of muscle mass associated to inflammatory states suggesting that leptin treatment may be an attractive therapeutic approach to prevent muscle loss associated with inflammatory diseases.

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Research Article

Effects of Sitagliptin Treatment on Dysmetabolism, Inflammation, and Oxidative Stress in an Animal Model of Type 2 Diabetes (ZDF Rat)

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The purpose of this paper is to evaluate the chronic effect of sitagliptin on metabolic profile, inflammation, and redox status in the Zucker Diabetic Fatty (ZDF) rat, an animal model of obese type 2 diabetes. Diabetic and obese ZDF (*fa/fa*) rats and their controls (ZDF +/+) were treated during 6 weeks with vehicle (control) and sitagliptin (10 mg/kg/bw). Glucose, HbA1c, insulin, Total-c, TGs, IL-1β, TNF-α, CRP*hs*, and adiponectin were assessed in serum and MDA and TAS in serum, pancreas, and heart. Pancreatic histology was also evaluated. Sitagliptin in diabetic rats promoted a decrease in glucose, HbA1c, Total-c, and TGs accompanied by a partial prevention of insulinopenia, together, with a decrease in CRP*hs* and IL-1β. Sitagliptin also showed a positive impact on lipid peroxidation and hypertension prevention. In conclusion, chronic sitagliptin treatment corrected the glycaemic dysmetabolism, hypertriglyceridaemia, inflammation, and hypertension, reduced the severity of the histopathological lesions of pancreatic endocrine and exocrine tissues, together with a favourable redox status, which might be a further advantage in the management of diabetes and its proatherogenic comorbidities.

1. Introduction

Type 2 diabetes mellitus (T2DM) is the most common endocrine disorder worldwide, affecting more than 200 million people [1]. Pathogenesis of this disease involves abnormalities in glucose and lipid metabolism, including inadequate insulin secretion from pancreatic β-cells and resistance to insulin activity (insulin resistance) [2].

Hyperglycaemia and hyperlipidaemia are the key promoters, through distinct mechanisms, of reactive oxygen species (ROS) and advanced glycation end products (AGEs) production, which causes cell damage and insulin resistance [3, 4]. Moreover, these high levels of glucose and lipids stimulate pro-inflammatory cytokines, promote lipid peroxidation, thus contributing to beta-cell degradation, particularly due to apoptosis pathways [5]. Actually, inflammation and oxidative stress play a major role in type 2 diabetes mellitus (T2DM) pathophysiology, contributing for obesity, insulin resistance and cardiovascular complications, which further aggravate the disease. However, so far, there are no therapeutic options able to efficiently act not only on the glucose control but, and specially, on the prevention of T2DM evolution and its complications, namely, by beta-cell function preservation.

In T2DM patients, the effect of the glucose-dependent insulinotropic polypeptide (GIP), as well as the secretion

of the glucagon-like peptide-1 (GLP-1), is diminished or absent, contributing to insulin secretion deficiency [6]. These two incretins are secreted by the intestine [7] and stimulate insulin secretion by beta-cells, in a glucose-dependent manner [8], preventing hypoglycemia. In animal models, continuous infusion of GLP-1 or injection of long-acting GLP-1 mimetics, such as exendin-4, has shown a remarkable glucose-lowering efficacy, together with an ability to increase beta-cell neogenesis and reduce apoptosis and alpha-cell glucagon secretion [9–11]. Despite the beneficial actions of GLP-1 and GIP, their use as antidiabetic agents (mimetics) is impractical due to their short half-lives, as a result of their rapid inactivation by dipeptidyl peptidase-IV (DPP-IV) [12, 13]. Thus, orally administered DPP-IV inhibitors have emerged as a new class of antihyperglycaemic agents with the ability for extending the biological effects of incretin hormones through the inhibition of their degradation [14, 15], with the advantage of higher stability and bioavailability when compared with the mimetics.

Sitagliptin, an orally available DPP-IV inhibitor developed to be used as a once daily treatment for T2DM, has shown beneficial effects on glycaemic control, reducing HbA1c, and preventing hypoglycemia, as well as on islet mass and function, with no relevant adverse effects [16, 17]. Considering the vast physiological actions promoted by the incretins, not only related with the control of glucose by insulin and glucagon regulation, but also with the peripheral insulin sensitization, cardiac and neuronal protection and beta-cell preservation, the use of an incretin enhancer (such as sitagliptin) might present beneficial effects on diabetes pathophysiology and on prevention of its serious complications, which deserves better elucidation.

The male Zucker Diabetic Fatty (ZDF) rat displays glucose intolerance, marked insulin resistance, and hyperlipidaemia, and becomes overtly diabetic after 8 weeks of age if fed a diet containing 6.5% fat [18]. In the prediabetic state, the male ZDF rat experiences a steady increase in basal insulinaemia and plasma free fatty acid (FFA) levels. Hyperglycemia develops between 8 and 10 weeks of age, leading to overt diabetes and collapsing insulin secretion [19]. This profile mimics the progressive loss of glucose-stimulated insulin secretion in human type 2 diabetes and, thus, the ZDF rat represents a good animal model for studying human T2DM pathophysiology and the effects of therapeutic options [20].

The purpose of this study was, thus, to assess the effects of chronic sitagliptin treatment on the metabolic profile, inflammation, and redox status and pancreas histology in the ZDF rat, an animal model of obese T2DM.

2. Material and Methods

2.1. Animals and Experimental Design. Male ZDF rats (ZDF/Gmi, *fa/fa*) and their littermates (ZDF/Gmi, +/+) were purchased from Charles River Laboratories (Barcelona, Spain) with 6 weeks of age. Rats were properly housed, handled daily, and kept at a controlled standard temperature (22-23°C), humidity (60%) and light-dark cycles (12/12 hours). Throughout the experiment, the animals were fed distilled water *ad libitum* and rodent maintenance chow (A-04 Panlab, Barcelona, Spain) containing 15.4% of protein and 2.9% of lipids). The chow was adapted to the animal's body weight (BW): 100 mg/g. Animal experiments were conducted according to the European Council Directives on Animal Care and to the National Laws.

When aged 20 weeks (T0), the diabetic ZDF (*fa/fa*) rats were divided in 2 subgroups ($n = 8$ rats each): a control and a treatment group, receiving, respectively, by oral gavage, once a day (6:00 PM), during 6 weeks, the vehicle (orange juice) and sitagliptin (10 mg/kg/BW/day). The same procedures were adopted with the lean nondiabetic ZDF (+/+) control rats. The ZDF (+/+) control group under sitagliptin treatment showed no relevant differences when compared with the ZDF (+/+) control rats under vehicle and, thus, the results were excluded from tables and figures in order to facilitate data comparison and interpretation.

Food intake and BW were measured each day before treatment and expressed as weekly average values. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were determined in conscious rats using a tail-cuff sphygmomanometer LE 5001 (Letica, Barcelona, Spain) in appropriate restriction cages. Pulse pressure (PP) was calculated by the difference between the systolic and the diastolic readings (PP=SBP−DBP). Blood pressure (BP) values, obtained by averaging 8 to 10 measurements, were recorded by the same person, in a similar peaceful environment. Measurements were performed at T0 and at the end of the study (Tf) with special precautions to minimize stress-induced fluctuations in BP, as previously described [21].

2.2. Sample Collection and Preparation. Blood: when aged 20 weeks (T0) and at the end of the experience (26 weeks - Tf) the rats were subjected to intraperitoneal anesthesia with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal) and blood samples were immediately collected by venipuncture from the jugular vein into syringes without anticoagulant (for serum samples) or with the appropriate anticoagulant: ethylene-diaminetetraacetic acid (EDTA)-2K for Glycosylated haemoglobin (HbA_1c) measurement.

The rats were sacrificed by anesthetic overdose. The pancreas and the heart were immediately removed, placed in ice-cold Krebs' buffer and Bock's fixative, respectively, and carefully cleaned of extraneous fat, lymph nodes and connective tissue. The organs were cross-sectioned and cryopreservated, fixed and processed for paraffin embedding in accordance with subsequent analysis protocols.

2.3. Glycaemic and Lipidic Profile Assays. Serum total cholesterol (Total-c) and triglycerides (TGs) were analysed on a Hitachi 717 analyser (Roche Diagnostics) using standard laboratorial methods. Total-c reagents and TGs kit were obtained from bioMérieux (Lyon, France). Serum glucose levels were measured using a Glucose oxidase commercial kit (Sigma, St. Louis, Mo, USA). Considering the variability of serum glucose levels in the rat, glycosylated haemoglobin

Table 1: Body weight, lipid profile and blood pressure in the control and diabetic ZDF rats at the initial and final time (6 weeks of vehicle or sitagliptin treatment).

	Initial Time (20 wks)		Final Time (26 wks)		
	Control ZDF (+/+)	Diabetic ZDF (*fa/fa*)	Control ZDF (+/+)	Diabetic ZDF (*fa/fa*)	
Groups	($n = 16$)	($n = 16$)	Vehicle ($n = 8$)	Vehicle ($n = 8$)	Sitagliptin ($n = 8$)
BW (g)	406.70 ± 6.83	388.10 ± 8.87	445.70 ± 8.16	354.40 ± 8.85^{aaa}	380.00±14.46
Total-c (mg/dl)	77.50 ± 1.50	155.50 ± 3.50^{aaa}	93.00 ± 2.96	193.00 ± 9.79^{aaa}	193.10±4.62
TGs (mg/dl)	115.00 ± 11.00	374.50 ± 4.95^{a}	154.00 ± 19.14	400.20 ± 27.00^{aaa}	237.10 ± 22.54^{bbb}
Systolic (mmHg)	115.50 ± 0.83	125.20 ± 0.27	116.00 ± 2.52	127.80 ± 1.23^{a}	101.60 ± 0.78^{bbb}
Diastolic (mmHg)	100.98 ± 0.82	91.46 ± 0.83	103.50 ± 1.94	112.70 ± 3.98	94.86 ± 0.70^{bbb}
Mean (mmHg)	104.25 ± 0.25	108.20 ± 1.42	104.30 ± 4.25	117.40 ± 3.04^{a}	96.86 ± 0.51^{bbb}
Pulse P (mmHg)	14.52 ± 0.98	33.74 ± 0.37	14.00 ± 4.16	15.09 ± 3.08	6.71 ± 1.11^{b}

BW, body weight; P, pressure; SITA, sitagliptin; Total-c, Total-cholesterol; TGs, triglycerides; ZDF, Zucker diabetic fatty. Values are means ± SEM of n rats. Comparisons between groups: a - ZDF (*fa/fa*) versus ZDF (+/+) and b - sitagliptin versus vehicle; $P < .05$, $P < .01$ and $P < .001$ for one, two or three letters, respectively.

(HbA_1c) levels were used as an index of glucose control, through the DCA 2000+ latex immunoagglutination method (Bayer Diagnostics, Barcelona, Spain). Plasma insulin levels were quantified by using a rat insulin Elisa assay kit from Mercodia (Uppsala, Sweden). Insulin sensitivity of individual animals was evaluated using the previously validated homeostasis model assessment (HOMA) index [21]. The formula used was as follows: [HOMA-IR] = fasting serum glucose (mmol/l) x fasting serum insulin (μU/ml)/22.5. The values used (insulin and glucose) were obtained after an overnight of food deprivation.

2.4. Inflammatory Profile and Redox Status. Serum levels of interleukin-1β (IL-1β), tumour necrosis factor α (TNF-α) and adiponectin were all measured by rat-specific Quantikine ELISA kits from R&D Systems (Minneapolis, USA). High-sensitive C-reactive protein (CRP*hs*) was determined by using a rat-specific Elisa kit from Helica Biosystems Inc. (Fullerton, CA, USA). All assays were performed according to the manufacturers' recommendations, in duplicate.

The thiobarbituric acid reactive-species (TBARs) assay was used to assess serum, pancreas and heart products of lipid peroxidation, via malondialdehyde (MDA), according to that previously described in [22]. Samples were analysed spectrophotometrically at 532 nm using 1,1,3,3-tetramethoxypropane as external standard. The concentration of lipid peroxides (in MDA) was expressed as μmol/l in the plasma and as μmol/g tissue in the pancreas and heart. Ferric reducing antioxidant potential (FRAP) assay was used to estimate serum total antioxidant status (TAS) [23].

2.5. Histological Studies. Specimens were paraffin-embedded and the 3 μm thick sections stained for routine histopathological diagnosis with haematoxylin and eosin (HE). All samples were examined by light microscopy using a Microscope Zeiss Mod. Axioplan 2. The degree of injury visible by light microscopy was scored in a single-blind fashion by the pathologist to the animal study group. Endocrine pancreatic damage was assessed by evaluating changes in the islets of Langerhans, namely the shape (architecture), presence of inflammatory infiltrate, fibrosis, vacuolization and intraislets congestion. A semiquantitative rating for each slide ranging from 0 (minimal) to 3 (severe and extensive damage) was assigned to each component. The exocrine pancreatic damage was evaluated, according to the presence of congestion, fibrosis, and inflammatory infiltrate in the interstitial tissues and graded, also, in the same semiquantitative rating.

2.6. Statistical Analysis. Results are shown as mean ± standard error of the mean (SEM). The comparison of values between groups was performed by using ANOVA followed by the Bonferroni post hoc test, through appropriate software (GraphPadPrism 5.0 from GraphPad Software Inc., La Jolla, CA, USA). Significance was accepted at a P less than .05.

3. Results

3.1. Effects of Chronic Sitagliptin Treatment on Body Weight and Glycaemic and Lipidic Profiles. Concerning the body weight, no significant differences were encountered between the diabetic and the lean control rats in the beginning of treatments (T0: week 20), despite the obese profile encountered in the diabetic ZDF (*fa/fa*) rats between the 8th and the 14th week (data not shown). At the end of the study (26 weeks), the control diabetic ZDF (*fa/fa*) rats exhibit an 8.7% reduction in their BW ($P < .001$); nevertheless, the lean control group gained weight. Sitagliptin treatment, during 6 weeks, stabilized the loss of weight in the diabetic ZDF *(fa/fa)* rats, even preventing part of the BW loss when compared with the rats without treatment (Table 1).

The determination of serum glucose, HbA1c, Total-c and TGs concentrations was carried at the initial time (T0: 20 weeks old) and at the end of the study (Tf: 26 weeks old). At the T0, the diabetic group showed a hyperglycaemic and a hyperlipidemic profile, also seen at the final time (Figure 1(a) and Table 1). As illustrated in Figure 1(b), the HbA1c values were higher in the diabetic rats than those of the control animals, confirming the glycaemic deregulation. The diabetic ZDF (*fa/fa*) rats have also presented higher levels of Total-c

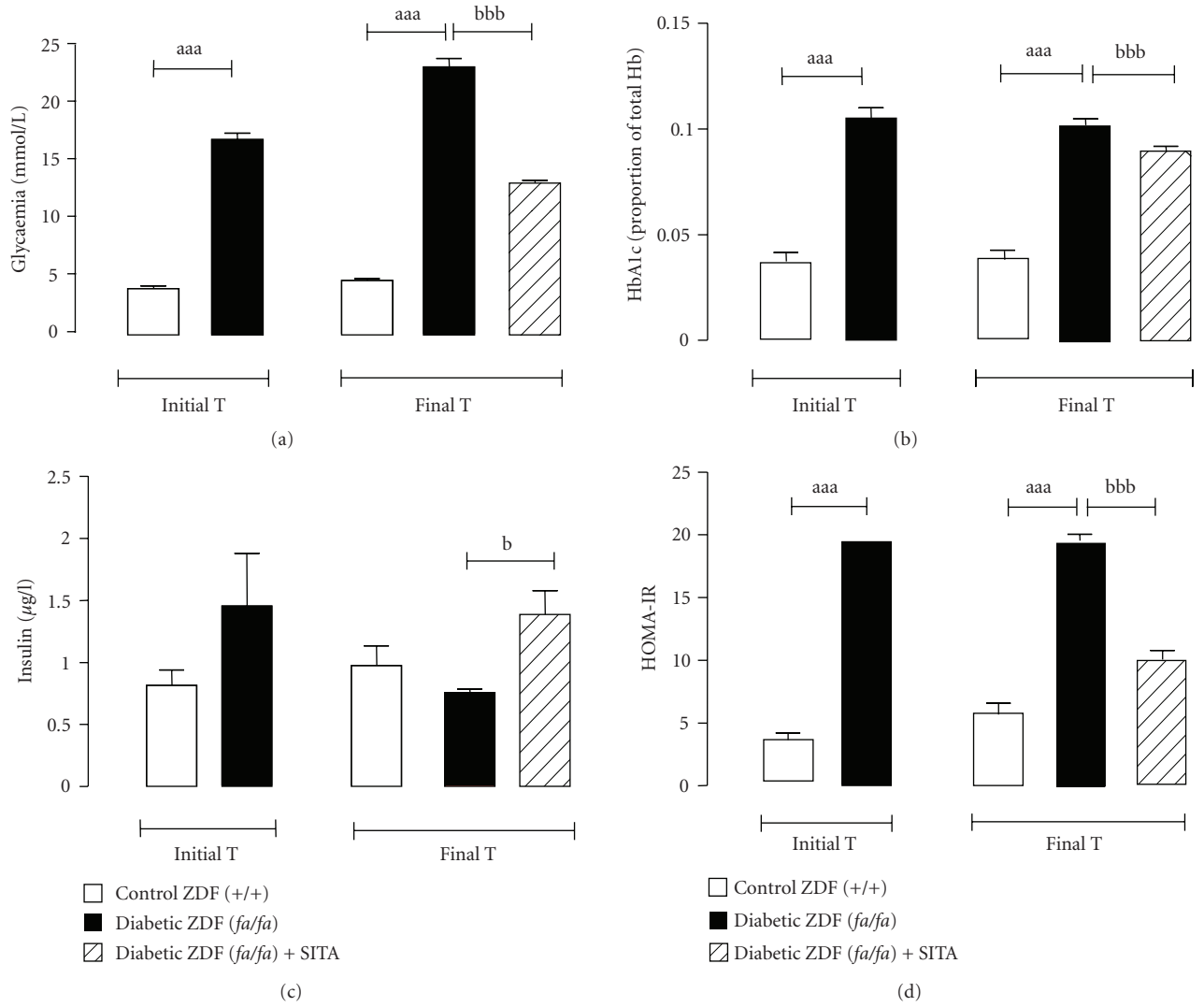

Figure 1: Glycaemic and insulinaemic profiles. Serum Glycaemia (a), HbA1c (b), insulinaemia (c) and insulin resistance (HOMA-IR) index (d), for the control (+/+) and diabetic (*fa/fa*) ZDF rats, in the initial and final times (6 weeks of vehicle or 10 mg/kg BW/day sitagliptin treatment). Comparisons between groups ($n = 8$ each): a - ZDF (*fa/fa*) versus ZDF (+/+) and b - with sita versus without sita; $P < .05$, $P < .01$ and $P < .001$ for one, two or three letters, respectively. HOMA-IR, homeostasis model assessment—insulin resistance.

and TGs versus the control ZDF (+/+) animals, in both times (Table 1).

After 6 weeks of sitagliptin treatment (Tf: 26 weeks), a significant improvement in glycemic control was observed in diabetic ZDF (*fa/fa*) rats (486.3 ± 19.1 mg/dl), when compared with the vehicle-treated diabetic animals (523.3 ± 15.6 mg/dl; $P < .001$) (Figure 1(a)). This pattern of changes is also expressed by the HbA1c levels, which decreased by 11.1% in sitagliptin-treated ZDF (*fa/fa*) rats when compared with the diabetic rats not treated with the drug (Figure 1(b)). TGs were significantly reduced (50%; $P < .001$) in the diabetic rats treated with sitagliptin during 6 weeks versus the diabetic vehicle-treated group (Table 1).

3.2. Effects of Chronic Sitagliptin Treatment on Insulin Levels and Insulin Resistance (HOMA-IR). At the beginning of the study (T0), insulin levels were higher in the diabetic rats than those of the control, but the differences did not reach statistical significance. At the final time, the vehicle-treated ZDF (*fa/fa*) rats exhibit relative insulinopenia (0.75 ± 0.05 μg/l), when compared to vehicle-treated ZDF (+/+) (1.05 ± 0.30 μg/l) (Figure 1(c)), accompanied by a significant augment ($P < .001$) of insulin resistance (HOMA-IR index) (Figure 1(d)). The elevation of insulin resistance was prevented ($P < .001$) in the sitagliptin-treated diabetic (*fa/fa*) rats (Figure 1(d)).

3.3. Effects of Chronic Sitagliptin Treatment on Blood Pressure. The vehicle-treated ZDF (*fa/fa*) group showed significantly ($P < .05$) higher levels of systolic and mean BP, together with a trend to higher diastolic and pulse pressure, when compared with the vehicle-treated ZDF (+/+) group. Sitagliptin

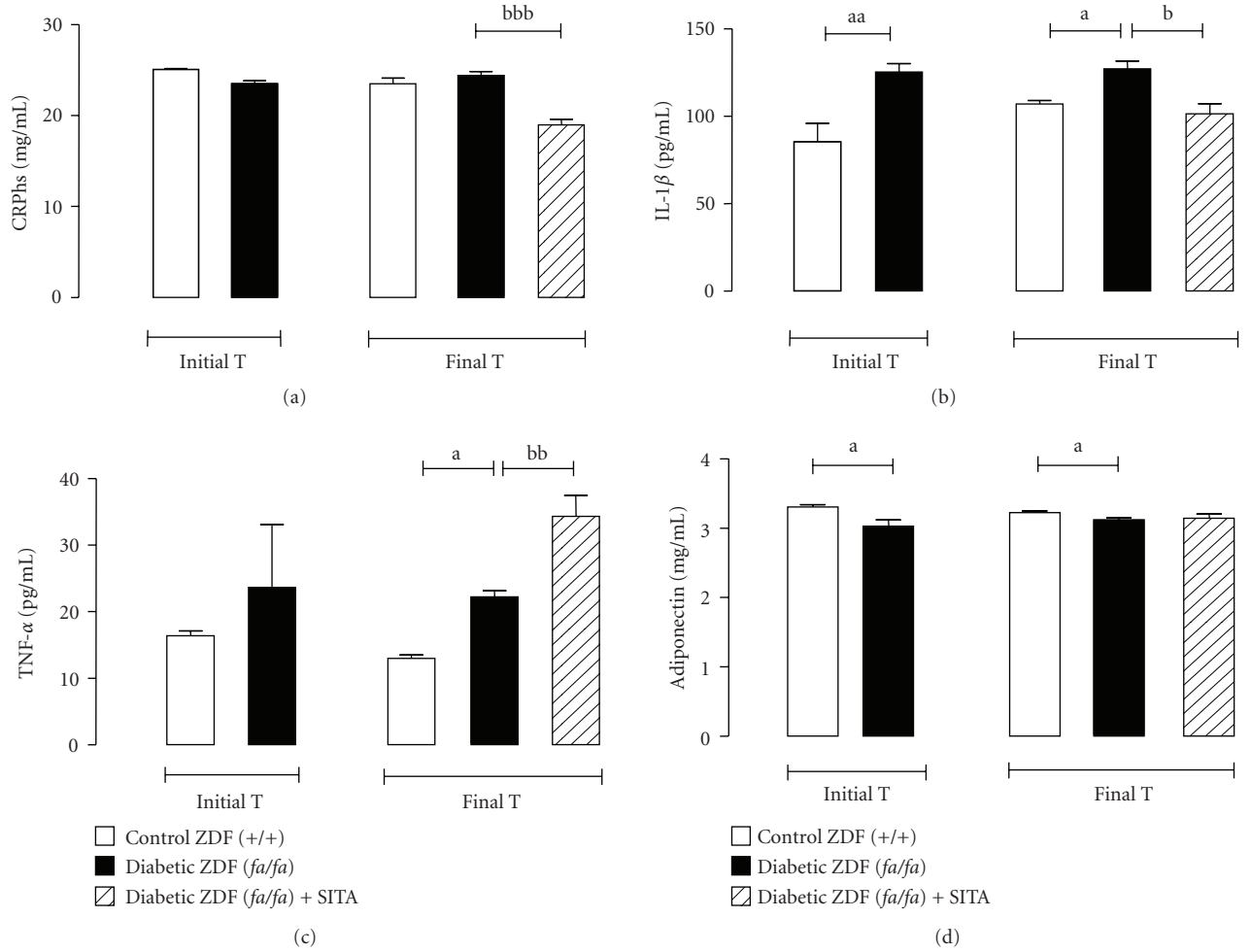

Figure 2: Serum inflammatory markers. Serum CRP*hs* (a), IL-1β (b), TNF-α (c) and Adiponectin (d) for the control (+/+) and diabetic (*fa/fa*) ZDF rats, in the initial and final times (6 weeks of vehicle or 10 mg/kg BW/day sitagliptin treatment). Comparisons between groups ($n = 8$ each): a - ZDF (*fa/fa*) versus ZDF (+/+) and b - with sita versus without sita; $P < .05$, $P < .01$ and $P < .001$ for one, two or three letters, respectively. CRP*hs*, high-sensitive C-reactive protein; IL-1β, interleukin-1beta; TNF-α, Tumor necrosis factor-alpha.

treatment has significantly prevented the blood pressure rise (hypertension) in the diabetic rats (Table 1).

3.4. Effects of Chronic Sitagliptin Treatment on Inflammatory Profile. Concerning the serum CRP*hs* levels, no significant differences were observed between the diabetic ZDF (*fa/fa*) and the nondiabetic ZDF (+/+) vehicle-treated groups (Figure 2(a)). However, there was higher serum levels of IL-1β and TNF-α and reduced of adiponectin in the vehicle-treated diabetic ZDF (*fa/fa*) rats when compared with the vehicle-treated nondiabetic (+/+) rats (Figures 2(b), 2(c) and 2(d)). Sitagliptin treatment has significantly decreased the levels of CRP*hs* ($P < .001$) and IL-1β ($P < .05$) in the diabetic ZDF rats (Figures 2(a) and 2(b)). However, the diabetic (*fa/fa*) animals under stagliptin therapy showed, at the end of the study, elevated ($P < .01$) levels of TNF-α (Figure 2(c)), without significant changes on serum adiponectin contents (Figure 2(d)).

3.5. Effects of Chronic Sitagliptin Treatment on Serum and Tissue Redox Status. The vehicle-treated diabetic ZDF (*fa/fa*) group exhibited significantly higher levels of serum MDA (at the T0 and Tf), accompanied by a compensatory elevation of TAS in the final time (Figures 3(a) and 3(b)). Sitagliptin treatment during 6 weeks has decreased ($P < .01$) serum TAS content, whereas there were no differences in serum MDA levels (Figures 3(a) and 3(b)). On the contrary, we observed a significant reduction of pancreas ($P < .001$) and heart ($P < .001$) MDA levels in the sitagliptin-treated diabetic ZDF (*fa/fa*) rats when compared with the vehicle-treated (*fa/fa*) rats (Figures 3(c) and 3(d)).

3.6. Effects of Chronic Sitagliptin Treatment on Pancreatic Histology. In the control rats (ZDF (+/+) under vehicle treatment, there was no pathological changes in the endocrine and exocrine pancreas (Figure 4(a)). Langerhans islets of diabetic ZDF animals treated with sitagliptin presented a

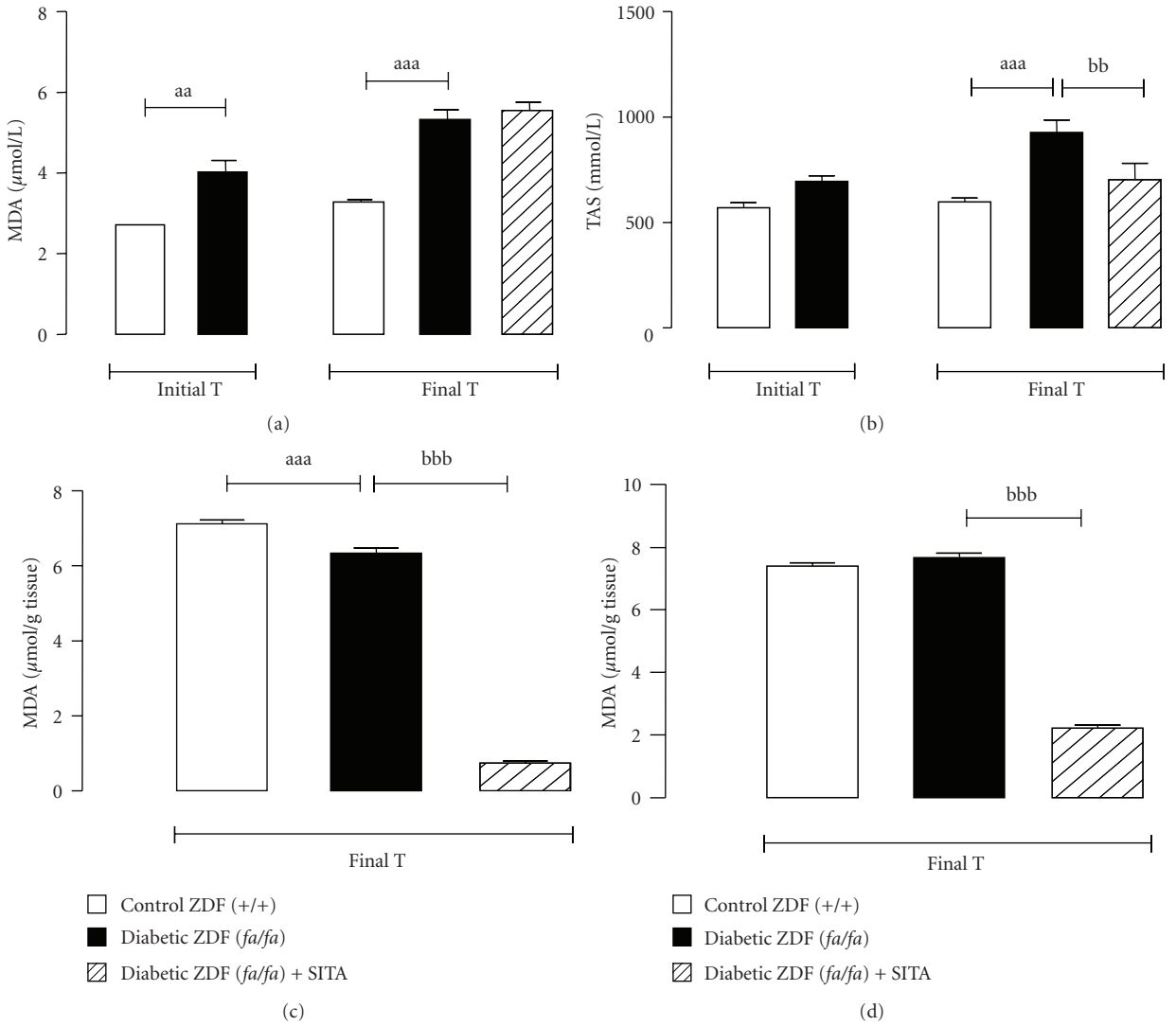

Figure 3: Serum and tissue redox status markers. Serum MDA (a) and TAS (b) and pancreas (c) and heart (d) MDA, for the control (+/+) and diabetic (*fa/fa*) ZDF rats, in the initial and final times (6 weeks of vehicle or 10 mg/kg BW/day sitagliptin treatment). Comparisons between groups ($n = 8$ each): a - ZDF (*fa/fa*) versus ZDF (+/+) and b - with sita versus without sita; $P < .05$, $P < .01$ and $P < .001$ for one, two or three letters, respectively. MDA, malondialdehyde; TAS, total antioxidant status.

diminution in fibrosis intensity (Figure 4(c)). While vehicle-treated diabetic ZDF *(fa/fa)* rats presented a higher number of animals in advanced degrees of fibrosis severity (75.0% of grade 3; 12.5% of grade 2 and of 12.5% grade 1), in the sitagliptin-treated group the severity of fibrosis rating ranged only from 1 to 2 (37.5% and 62.5%, resp) (Figures 4(a) and 4(b)). An amelioration of the inflammatory infiltrate in the endocrine pancreas was encountered when the diabetic ZDF rats were cronically treated with sitagliptin (Table 2). The treated group presented 87.5% rats with grade 1 inflammatory infiltrate, whereas in the vehicle-treated group all rats presented inflammatory infiltrate (37.5% of grade 3 and 62.5% of grade 2). Intra-islet cellular grade 2 vacuolation was present in most of the rats (75%) without treatment (vehicle-treated group). This grade was quantitatively reduced in the treated group, in which only 1 rat (12.5%) presented grade 2 vacuolation, representing the remainder (37.5%) a grade 1 vacuolation. Congestion affected one vehicle-treated diabetic ZDF (*fa/fa*) rat, being completely absent in the sitagliptin group (Table 2). Nevertheless, on the parenchymal structure or islet size, only subtle differences were broadly detected.

All the diabetic ZDF (*fa/fa*) rats without stagliptin treatment exhibited in the exocrine pancreas a variable degree of fibrosis and ductal hypertrophy rating in levels 1, 2 and 3, as shown in Table 2 (Figures 4(d) and 4(e)). All the rats presented inflammatory infiltrate rating from 1 (37.5%) to 2 (62.5%). A grade 2 congestion was observed in most of the vehicle-treated rats (75.0%). Lesions of the exocrine pancreas of diabetic rats chronically treated with sitagliptin, when compared with those without treatment, exhibited a decrease in fibrosis, being absent in most of the animals (62.5%), with the remaining cases showing fibrosis rating in

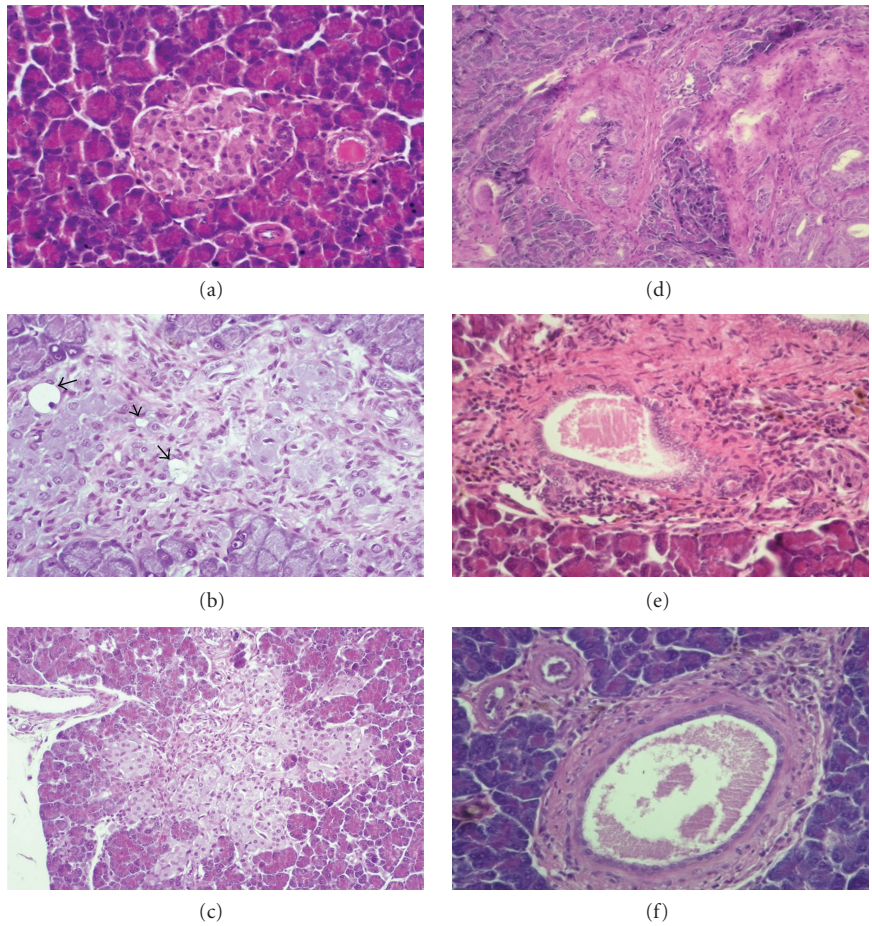

Figure 4: Pancreatic histology at the end of experimental period. Endocrine pancreas (a, b and c): (a) Typical islet from control ZDF (+/+) rats under vehicle treatment, without changes in the endocrine and exocrine pancreas; (b) Extensive fibrosis, vacuolation and loss of architecture in diabetic ZDF (*fa/fa*) rats under vehicle treatment; (c) Diminution in fibrosis intensity and vacuolation in Langerhans islet from diabetic ZDF (*fa/fa*) rats treated for 6 weeks with 10 mg/kg BW/day of sitagliptin, between weeks 20 and 26 (final time); Exocrine pancreas (d, e and f): (d) Severe fibrosis (III) with *neocanaliculi* (original magnification x 200) and (e) Congestion and intense inflammatory infiltrate from diabetic ZDF (*fa/fa*) rats treated with vehicle; (f) Marked decrease in fibrosis severity from diabetic ZDF (*fa/fa*) rats treated with sitagliptin. hematoxylin and eosin staining (original magnification x 400).

grade 1 and 2 (Figure 4(f)). Despite the presence of grade 1 or 2 (each representing 50%) inflammatory infiltrate in all rats, a reduction in severity was found in one of the animals (Table 2). The severity of congestion suffered a decrease from level 2 to level 1 in 50% of the rats and was completely absent in the other 50% of the group.

4. Discussion

Previous reports suggest that local and systemic low-grade inflammation and oxidative stress, which are mainly fuelled by hyperglycaemia and hyperlipidaemia, are important mediators of beta-cell degradation, insulin resistance and T2DM complications in many individuals [24–26]. It is now recognized that adipocytes, particularly those located within the visceral fat, are major secretors of both pro-and antiinflammatory factors, often referred to as adipokines [27, 28]. Several well-known markers of inflammation secreted by the adipose tissue, including IL-6 (which stimulated the hepatic synthesis of CRP), IL-1β and TNF-α, have been referred as independent predictors of diabetes [28–30]. Adiponectin, an adipokine, has demonstrated antiinflammatory properties, protection against insulin resistance, as well as against the development of atherosclerosis [31–34].

In this study, we assessed the effects of chronic sitagliptin treatment on glucose and lipids deregulation and on other cardiometabolic risk factors in an animal model of obese type 2 diabetes mellitus, the ZDF rat. Since the diagnosis of the disease is frequently late, when diabetes pathophysiological mechanisms are already advanced and the complications have already been initiated, we chose to use the diabetic ZDF rats in an established diabetes stage, which, according to our previous data, is when the animals aged 20 weeks [35, 36].

Concerning the ZDF model of type 2 diabetes, our results have demonstrated the key features encountered in type 2 diabetes patients. Therefore, at the beginning of

Table 2: Number of rats exhibiting the different pathology scores observed in endocrine (A) and exocrine (B) pancreas.

A-Endocrine pancreas lesions																
Evaluated parameters	Inflammatory Infiltrate				Fibrosis				Intra islet Vacuolation				Congestion			
Score	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Groups ($n = 8$, each)																
ZDF (+/+) vehicle	7	1	0	0	6	2	0	0	8	0	0	0	8	0	0	0
ZDF (*fa/fa*) vehicle	0	0	5	3	0	1	1	6	2	0	6	0	7	0	1	0
ZDF (*fa/fa*) sitagliptin	1	7	0	0	0	5	3	0	4	3	1	0	8	0	0	0
B – Exocrine pancreas lesions																
Evaluated parameters	Inflammatory Infiltrate				Fibrosis								Congestion			
Score	0	1	2	3			0	1	2	3			0	1	2	3
Groups ($n = 8$, each)																
ZDF (+/+) vehicle	8	0	0	0			7	1	0	0			8	0	0	0
ZDF (*fa/fa*) vehicle	0	3	5	0			0	3	3	2			2	6	0	0
ZDF (*fa/fa*) sitagliptin	0	4	4	0			6	1	1	0			4	4	0	0

ZDF, Zucker diabetic fatty.

the study (initial time: 20 weeks of age) the diabetic rats presented hyperglycaemia, hypercholesterolaemia, hypertriglyceridaemia, increased HbA1c and hyperinsulinaemia, accompanied by insulin resistance (HOMA-IR). Insulin levels of ZDF (*fa/fa*) rats were already decreased when compared with the controls, indicating an impaired insulin secretion by the pancreatic beta-cell. Furthermore, the ZDF (*fa/fa*) rats presented obesity between the 8th and the 14th week of age (data not shown), but started losing weight until the week 20. This BW decrease continued throughout the experimental period, which might be viewed as a complication of diabetes. Furthermore, the ZDF diabetic rats also presented, when compared with the nondiabetic ZDF (+/+) controls, a pro-inflammatory profile, represented by the reduced content of the antiinflammatory adipokines, adiponectin, and the increased level of the pro-inflammatory cytokines IL-1β and TNF-α. However, we should identify two surprising aspect encountered in the diabetic ZDF (*fa/fa*) rats at 20 weeks-old, which contrasts with previous data from us concerning the characterization of this animal of obese type 2 diabetes [35, 36], that were related to the almost unchanged serum CRP*hs* levels between the diabetic and the control (nondiabetic) animals and the only slightly (but significantly) lower adiponectin in the ZDF diabetic rats, suggesting that inflammation at this point (week 20) was more closely related with other players (such as TNF-α and IL-1β) and, as well, that the BW loss (which might represent an pathophysiological aggravation of the disease) might change the pattern of the inflammatory profile.

At the end of the experience, week 26, the ZDF rats aggravated their diabetic state, viewed by a higher hyperglycaemia, accompanied by increased HbA1c, insulin resistance and reduced plasma concentration of insulin, suggesting that the relative insulinopenic state, which started at the beginning of the study, was aggravated. Moreover, the ZDF diabetic rats continue to lose weight and showed an aggravated hypercholesterolaemia, hypertriglyceridaemia, together with inflammation and hypertension. At this time, however, the increased serum MDA content was accompanied by a compensatory increase in serum TAS, which might explain the unchanged values of tissue (pancreas and heart) MDA between the diabetic and nondiabetic animals. In any case, between the week 20, corresponding to an established diabetes state, and the week 26, the diabetic rats aggravates the disease (viewed mainly by the aggravated hyperglycaemia and the insulinopenia) and its complications (hypertension), which is in agreement with our previous data concerning the metabolic characterization of this model of type 2 diabetes mellitus (the ZDF rat) [35, 36].

During the course of the study, the diabetic rats treated once a day with an incretin enhancer, the DDP-IV inhibitor sitagliptin, showed a remarkable beneficial effect on several important parameters, not only those related to the glycaemic control, as should be expected when using an antihyperglycaemic agent, but also on other cardiometabolic perturbations and complications related to diabetes. Therefore, chronic sitagliptin treatment has promoted a reduction of glucose and HbA1c levels, together with a partial correction of insulin reduction and an improvement of insulin resistance (HOMA-IR), which is in agreement with other reports [37, 38]. Furthermore, the reduction of BW was prevented and the hypertriglyceridaemia corrected, which was accompanied by a prevention of diabetes-induced hypertension, as previously suggested by other authors in [38, 39]. Future studies from us will estimate the effects of this DPP-IV inhibitor on the enzyme activity/expression, as well as on levels of GLP-1 and glucagon, in order to have a more detailed picture of how the incretins pathway is affected and its relative contribution for the effects of sitagliptin here reported.

Evaluation of endocrine pancreatic tissue suggests amelioration in Langerhans islets by sitagliptin treatment. In the exocrine pancreas an improvement in sitagliptin-treated rats was also observed. However, results must be carefully interpreted because they superimpose on those lesions presented by diabetic rats without treatment as result of obesity and/or type 2 diabetes. Matveyenko et al. (2009) using HIP rats reported beneficial effects of sitagliptin in endocrine pancreas, together with haemorrhagic pancreatitis in one sitagliptin treated rat, ductal metaplasia in three sitagliptin-treated rats and increased ductal proliferation in all sitagliptin-treated rats, suggesting chronic pancreatitis [40]. Nevertheless, they use a dosage 20 fold larger, with a duration of treatment twice longer than the one used in our present work. Despite the difference in rat specie, dose and route of administration, the studies of Matveyenko et al. (2009), using a DPP IV inhibitor, and of Nachnani et al. (2010) [41], using an injection of GLP-1 agonist to enhance endogenous GLP-1 levels, raise the possibility that the enhancement of endogenous GLP-1 levels could induce undetected low grade asymptomatic chronic pancreatitis. Despite the lower dose used, we observed beneficial effects of sitagliptin on metabolic profile and reduction in inflammatory markers, as well as an amelioration of fibrosis, vacuolization and congestion in endocrine pancreas. Others have observed similar results using FE 999011, an inhibitor of DPP IV, administrated orally in a dose of 10 mg/kg BW once a day [42]. The therapeutic dosage required to improve glucose tolerance, on an acute scale in humans (0.2 mg/kg), is 200-fold lower than the one used in the present study [43]. Our findings suggest that the compensatory change in circulating DPP-IV levels could be avoided by once-daily treatment and/or a lower inhibitor dosage.

Concerning the markers of inflammation and oxidative stress, this study demonstrated an important effect of sitagliptin on CRP*hs* and IL-1β serum levels, reducing the higher levels encountered in the diabetic rats. The effects on these mechanisms have contrasted with those encountered on TNF-α and adiponectin, in which an increment and the absence of influence, respectively, were observed, suggesting that distinct mechanisms regulates the different cytokines produced by the adipocyte tissue. The increment on serum TNF-α levels might eventually suggest undesirable side effect of sitagliptin. Therefore, it is well known that the inhibition of the serine protease DPP-IV in type 2 diabetes treatment prevents its activation of insulin-releasing peptide hormones. However, DPP-IV also cleaves many other molecules, including chemokines, suggesting that inhibition of this enzyme could have undesired side effects and might be responsible for allergic reactions and runny or stuffy nose, sore throat, and upper respiratory infection, described as sitagliptin side effects [44].

The beneficial effect on systemic CRP*hs* and IL-1β was accompanied by an improvement of tissue redox status, with a remarkable positive impact on lipid peroxidation in both the pancreas and the heart. These effects, together with a decrease in TGs content, might contribute to reduce pancreatic beta-cell deterioration, which is a feature of diabetes evolution to high deregulated states, and to alleviate the cardiovascular complications that accompany the evolution of the disease and that are responsible for the associated high mortality and morbidity rates worldwide [45]. The blood pressure amelioration found in our study might be secondary to the improvement of glucose and lipidic dysmetabolism, low-grade inflammation and oxidative stress status, which are factors undoubtedly linked with the cardiometabolic complication associated with diabetes. However, a direct favourable influence of sitagliptin on the cardiovascular system might occur, as suggested by the positive impact on heart redox status. Furthermore, the previously suggested antiapoptotic effect of the incretin modulators on the pancreas might be extended to other tissues, such as the heart. This hypothesis should be further reinforced in future studies. An adequate treatment for type 2 diabetes, according to the guidelines, should be focused not only on glycaemia control, but also, on reduction of triglycerides and blood pressure, thus preventing the cardiovascular complications [46–48]. According to previous data, there is yet no sufficient clinical data to assess the real influence of incretin modulators on cardiovascular disease prevention and on long-term cardiovascular safety [49, 50].

Several reports have indicated that DPP-IV inhibitors are as antihyperglycaemic as any other oral antidiabetic drugs, with the additional benefit of not promoting hypoglycaemia and weight gain [45]. Further studies, using another antidiabetic agent from other group, should be performed in order to confirm if the beneficial effects now obtained are clearly directly attributed to the mechanism of action of this compound and are not exclusively resulting from the improvement of glycemic control. Since GLP-1 receptors have been identified in several tissues related with the cardiovascular system, such as the cardiomyocytes and vascular endothelial cells, the effects of the incretin-based therapies, such as the DPP-IV inhibitors, point to a potential benefit on attenuation of type-2 diabetes-induced cardiovascular complication [45]. However, the current limitations are related to the lack of log-term clinical studies [49, 50]. In any case, considering the interesting properties demonstrated by these new class of antidiabetic agents, which make them different from the traditional drugs, and if the clinical studies are able to confirm other influences, apart the already reported glycaemic control and HbA1c reduction, in a near future their place in the treatment algorithm might be reviewed. Therefore, if the beneficial effects on beta-cell function preservation, as well as on prevention of diabetic complications, will be further confirmed, they might be recommended not only as adjuvant therapy when other antidiabetics fail to control glycaemia and HbA1c levels, but also, as one of the main choices for type 2 diabetes management and prevention of complications.

5. Conclusions

This study, using a model of obese T2DM (the ZDF rat), demonstrated that chronic inhibition of DPP-IV by sitagliptin can correct the glycaemic dysmetabolism, hypertriglyceridaemia, inflammation and hypertension, reduce severity of histopathological lesions of endocrine and

exocrine pancreas, jointly, with a favourable influence on the pancreas and heart lipid peroxidation, which have been identified as the key pathophysiological mechanism underlying insulin resistance, beta-cell degradation and associated micro-and-macrovascular complications. These influences here reported may become further advantages in the therapeutics of type 2 diabetes and in the prevention/management of its pro-atherogenic macrovascular complications.

Declaration of Interest

The authors report no conflict of interest.

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Review Article

Adipocytokines in Atherothrombosis: Focus on Platelets and Vascular Smooth Muscle Cells

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Visceral obesity is a relevant pathological condition closely associated with high risk of atherosclerotic vascular disease including myocardial infarction and stroke. The increased vascular risk is related also to peculiar dysfunction in the endocrine activity of adipose tissue responsible of vascular impairment (including endothelial dysfunction), prothrombotic tendency, and low-grade chronic inflammation. In particular, increased synthesis and release of different cytokines, including interleukins and tumor necrosis factor-α (TNF-α), and adipokines—such as leptin—have been reported as associated with future cardiovascular events. Since vascular cell dysfunction plays a major role in the atherothrombotic complications in central obesity, this paper aims at focusing, in particular, on the relationship between platelets and vascular smooth muscle cells, and the impaired secretory pattern of adipose tissue.

1. Introduction

Subjects affected by central obesity (i.e., by intra-abdominal fat excess) are characterized by insulin resistance, metabolic disorders, and vascular abnormalities which cooperate to induce high cardiovascular risk [1–3].

The so-called "metabolic syndrome", defined on the basis of a combination of central obesity, glucose intolerance, atherogenic dyslipidemia, and arterial hypertension [3, 4], is present in the large majority of these subjects.

In central obesity, abnormalities in the extent and distribution of fat mass are associated with a peculiar dysfunction of adipose tissue, responsible—together with the insulin resistance—of alterations of vascular function (including endothelial dysfunction), pro-thrombotic tendency, low-grade chronic inflammation, and oxidative stress: these defects, frequently associated as a cluster, represent the main pathogenetic link between obesity and the increased risk of athero-thrombotic events [5–7].

Omental adipose tissue, which comprises both adipocytes and a stromovascular cell fraction, is not an inert lipid storage site, but a dynamic endocrine organ able to synthesize and secrete many bioactive peptides—collectively named "adipocytokines"—deeply involved in the metabolic, vascular, and immunological homeostasis by paracrine and endocrine mechanisms [2, 6, 8–10].

Some molecules, directly synthesized by adipocytes and called "adipokines" (i) control energy balance and appetite, and influence insulin sensitivity via endocrine mechanisms, and (ii) modulate adipocyte size/number and angiogenesis via paracrine mechanisms, thus exerting a major role in the regulation of fat mass [10, 11]. Furthermore, they can also exert a role in the control of blood pressure, lipoprotein metabolism, coagulation, immunity and inflammation [5, 11].

Other peptides belonging to the cytokine group—produced and released by the stromal vascular components of adipose tissue (i.e., lymphocytes, fibroblasts, macrophages, endothelial cells, and preadipocytes) [8, 9, 12–14]—are mainly involved in local and systemic inflammation [8, 13–16]. The increase in abdominal adipose tissue mass dysregulates both adipokine and adipocytokine secretion patterns [10, 14].

With the exception of the insulin sensitizing peptide adiponectin, adipokine production and secretion are increased in central obesity [10, 11, 15]: this fact plays a pivotal role both in the pathogenesis of cardiovascular damage through adverse effects on hemostatic balance and vascular function [5, 6, 11], and in the amplification of inflammatory processes in vascular and nonvascular tissues [11, 13, 15].

Also, cytokine release is enhanced: this phenomenon is attributable to increased prevalence of hypertrophied adipocytes with altered adipokine synthesis and secretion, local hypoxia, as well as activation of resident inflammatory cells and macrophages [14, 15].

In particular, adipose tissue from individuals with central obesity synthesizes and releases increased amount of

(i) proinflammatory chemokines and cytokines, such as Monocyte Chemoattractant Protein-1 (MCP-1), macrophage migration inhibitory factor (MIF), tumor necrosis factor-α (TNF-α), and interleukins, including interleukin-1β (IL-1β) and interleukin-6 (IL-6) [15];

(ii) procoagulant and proinflammatory mediators such as tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) [15];

(iii) vasoactive substances such as angiotensinogen and endothelin-1 (ET-1) [17, 18];

(iv) molecules involved in the pathogenesis of insulin resistance, such as TNF-α and resistin [8, 10, 13–16].

Since central obesity is characterized by an enhanced cardiovascular risk, and it is known that dysfunctions of platelets and vascular smooth muscle cells (VSMC) are deeply involved in athero-thrombosis [19–22], the purpose of this review is to identify the actions of adipokines and adipocytokines on these cells, focusing in particular on the role of inflammation.

2. Role of Platelets in Thrombosis and Inflammation

Platelets are anuclear cell fragments released from megakaryocytes, hematopoietic cells that differentiate and undergo endomitosis [23, 24]. Despite the lack of nucleus, they contain mRNA and spliceosomal components for mRNA processing, as well as the translational machinery for protein synthesis [23, 25]: this justifies *de novo* synthesis by platelets of different mediators involved in the regulation of inflammatory and coagulant pathways including IL-1β, PAI-1, and TF [26, 27].

As evidenced in Figure 1, platelets play a pivotal role in the response to vascular injury through adhesion to exposed subendothelial layer triggered by different collagen types and adhesive proteins such as von Willebrand factor (vWF), fibronectin, laminin, fibulin and thrombospondin [26–29]. Activation of platelets by components of the subendothelial matrix is linked to exposure of membrane glycoprotein receptors including GPIb/V/IX complex which interacts with vWF, integrin α IIbβ_3 (GPIIb/IIIa) able to bind Arg-Gly-Asp [RDG] domain of vWF and fibrinogen, and GPVI which ensures a stable anchorage with subendothelial matrix by direct interaction with collagen [26–30]. Platelet activation and formation of aggregates are triggered also by thrombin [31], endogenous mediators released from storage granules and erythrocytes such as adenosine 5-diphosphate (ADP), and *de novo* synthesis of platelet activating factor (PAF), and thromboxane A_2 (TXA_2) [30, 32–34].

As shown in Figure 2, activated platelets also release inflammatory mediators from granules, including platelet-derived growth factor (PDGF) and platelet factor 4 (PF-4) [35–37].

Beyond acute activation as a consequence of vascular injury, circulating platelets are actively involved in all phases of the atherogenetic process, from atherosclerotic plaque formation to plaque inflammation and rupture [28, 37–39]. In these conditions, platelet reactivity is increased by reactive oxygen species (ROS) produced as a consequence of oxidative stress, by reduction of endothelial antithrombotic properties and by the increased availability of proinflammatory mediators, such as cytokines and chemokines [40, 41].

Actually, platelets release several mediators linking thrombosis and vascular inflammation such as the RANTES (regulated on activation, normal T-cell expressed and secreted) chemokine, PDGF, PF-4, Transforming growth factor-β (TGF-β), CD40 ligand (CD40L, CD154), P-selectin, and TXA_2 [35–37, 41].

RANTES recruits monocytes and T cells; in conjunction with P-selectin this chemokine can be immobilized on inflammed endothelial cells, thus inducing monocyte arrest and migration [41].

PDGF, the major growth factor contained in platelets [36–38], stimulates both migration and proliferation of VSMC by co-operation with serotonin, and TGF-β [36, 42, 43], and is chemotactic for monocytes [38, 43]; its effects on VSMC are critical for the development of atherosclerotic process [27, 37, 38].

PF-4—a member of the C-X-C chemokine subfamily—exerts chemotactic effects on monocytes, promotes monocyte-to-foam cell differentiation [27, 35, 36], enhances the binding of oxidized low density lipoproteins (LDL) to vascular wall, and inhibits LDL degradation through the LDL receptors [35, 44].

CD40L is a trimeric protein structurally related to TNF-α superfamily stored in the α -granules of resting platelets [45–49]. After platelet activation, it is rapidly exposed on cell surface and cleaved to release the soluble fragment (sCD40L) able to increase the stability of new platelet aggregates [49], and to activate vascular inflammatory mechanisms by inducing production of ROS, enhancing expression of adhesion molecules Vascular Cell Adhesion Molecule-1 (VCAM-1), Intercellular Adhesion Molecule-1 (ICAM-1), and E-selectin in endothelial cells and VSMC, and increasing secretion of cytokines, chemokines, matrix metalloproteinases (MMPs), and procoagulant factors [45–48].

Several cell types—including endothelial cells, VSMC, monocytes, neutrophils, B cells and fibroblasts—bind CD40L through the specific receptor CD40 [45, 50]. This

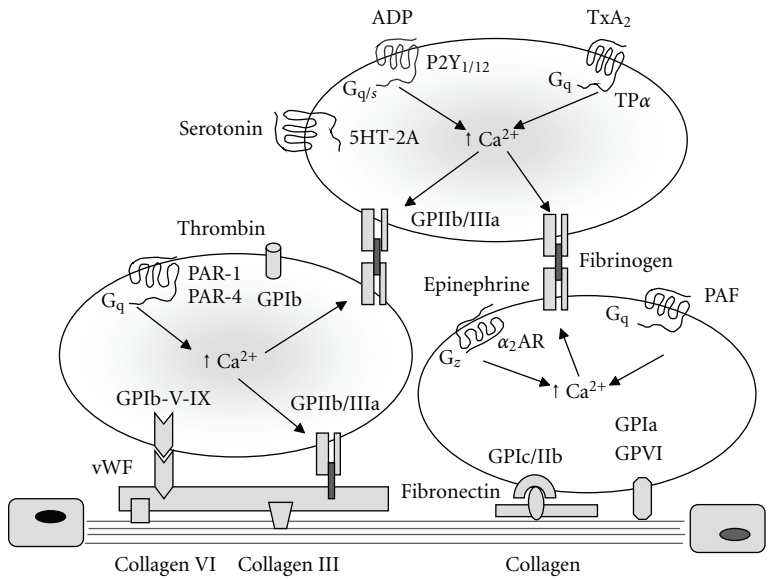

Figure 1: Mechanisms involved in platelet adhesion to subendothelial layer and activation. The diagram illustrates the role of von Willebrand factor (vWF), collagen and other proteins in platelet adhesion by linking to exposure of membrane glycoprotein receptors including GPIb//V/IX complex, GPIIb/IIIa, and GPVI which ensures a stable anchorage with subendothelial matrix by interaction with collagen. Platelet activation and aggregation are triggered by thrombin, endogenous mediators released from storage granules, and synthesis of platelet activating factor (PAF), and thromboxane A_2 (TXA_2). P2Y1/12, purinergic P2Y receptors; TPα, thromboxane α receptor; 5HT-2A, serotonin (5-hydroxytryptamine)-2A receptor; PAR-1, protease-activated receptor-1; PAR-4, protease-activated receptor-4; α_2 AR, α_2 adrenoreceptor.

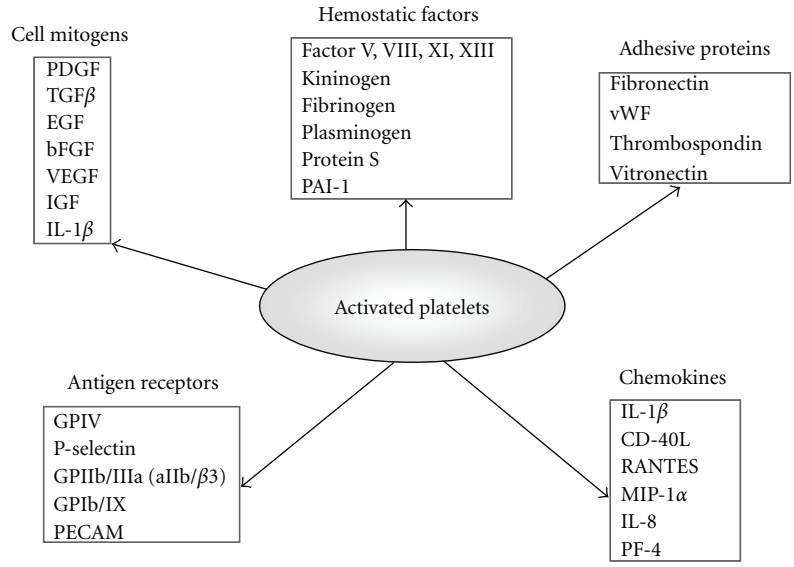

Figure 2: Platelet components involved in the coagulation cascade and the atherosclerotic process. PDGF, platelet-derived growth factor; TGFβ, transforming growth factor β; EGF, endothelial growth factor; bFGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; IL-1β, interleukin-1β; PAI-1, plasminogen activator inhibitor 1; vWF, von Willebrand factor; GP, glycoproteins; PECAM, platelet and endothelial cell adhesion molecule; CD40L, CD40 ligand (CD154); RANTES, regulated on activation, normal T-cell expressed and secreted; MIP-1α, macrophage inflammation protein 1α; IL-8, interleukin-8; PF4, platelet factor 4.

receptor has been recently identified also in adipocytes where it plays a relevant role in the crosstalk with resident lymphocytes [51]; furthermore, circulating sCD40L levels have been found at abnormally high levels in patients with obesity, type 2 diabetes mellitus and atherosclerotic vascular diseases [52–55].

P-selectin is stored in platelet α -granules [36] and, after activation, rapidly translocated upon cell surface becoming accessible to circulation; this phenomenon strengthens initial rolling contact between platelets and vessel wall and promotes RANTES deposition on endothelial cells, thus increasing monocyte recruitment [27–29, 41, 47, 56].

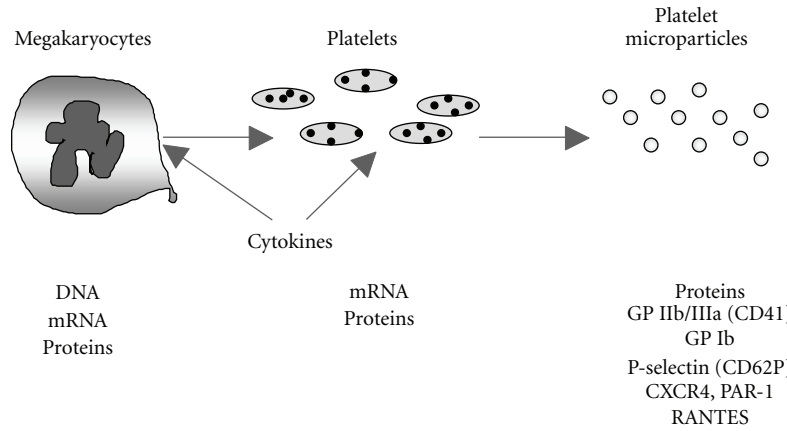

Figure 3: General features of circulating platelet microparticles (PMPs). PMPs are phospholipid microvesicles of 0.1–1 micron in size, sheded from parental cell fragments after stimulation with physiological agonists such as thrombin or collagen or exposure to shear stress (i.e., in severe stenosis). PMPs express functional adhesion receptors, including GPIIb/IIIa (CD41), P-selectin (CD62P), CXCR4, and PAR-1 and contain different proteins and coagulation factors, thus exerting a role in the hemostatic response and in the interplay between coagulation and inflammation. PMPs also simulate cytokine release and adhesion molecule expression in endothelial cells and contraction of VSMC. Elevated levels of circulating PMPs have been described in patients with arteriosclerosis, acute vascular syndromes, diabetes mellitus, as well as central obesity. GPIIb/IIIa, glycoprotein IIb/IIIa; GP Ib, glycoprotein Ib; CXCR4, chemokine (C-X-C motif) receptor 4; PAR-1, protease-activated receptor-1; RANTES, regulated on activation, normal T-cell expressed and secreted.

Platelet stimulation by agonists or exposure to high shear stress leads to formation and release of platelet-derived membrane-coated vesicles termed platelet microparticles (PMPs) [57, 58], which influence the activities of other cell types both regionally and systemically [58]. As evidenced in Figure 3, PMPs are lipid-protein complexes with a diameter $< 1\,\mu$ m, composed by vesicular fragments of the plasma membrane and α -granules [59]: their protein content plays a relevant role in both hemostasis and inflammation, by facilitating coagulation, promoting platelet and leukocyte adhesion to the subendothelial matrix, supporting angiogenesis and stimulating VSMC [58–60]. These effects may contribute to the chronic inflammatory state which characterizes atherosclerosis [58]; in particular, a portion of platelet-derived IL-1β associated with PMPs stimulates the production of chemoattractant molecules and cytokines and the expression of specific adhesion molecules in endothelial cells, thus enhancing their interaction with circulating leukocytes and, therefore, their ability to trigger inflammatory responses [61, 62]. Also the chemokine RANTES is delivered to sites of endothelial injury via PMPs to promote monocyte recruitment [56, 63].

3. Alterations of the Platelet Function in Central Obesity

Platelet hyperactivity is deeply involved in the increased atherothrombotic risk of patients affected by central obesity and type 2 diabetes mellitus [20, 21, 64].

A variety of defects of platelet function—mainly related to increased adhesiveness and activability *in vivo* and reduced sensitivity to physiological antagonists—has been identified in central obesity, as recently reviewed [20, 21].

Mean volume of circulating platelets—a parameter directly related to *in vivo* platelet activation [65] relevant to predict myocardial infarction occurrence and mortality and restenosis following coronary angioplasty [66]—is increased in obese patients, independently of the presence of other cardiovascular risk factors [66, 67]; furthermore, a positive correlation between body mass index (BMI) and mean platelet volume (MPV) has been found in obese individuals [66–69], whereas weight loss may lead to a decrease in platelet size and reactivity [68].

Increasing evidences indicate that the size of circulating platelets deeply influences their hemostatic potential being the response of larger platelets to aggregating stimuli more rapid and the amount of released mediators increased [65, 70].

Platelet volume, as well as other platelet parameters, are mainly determinated during megakaryocyte fragmentation in bone marrow [71, 72]. Even though the mechanisms influencing magakaryocytopoiesis are not completely understood, an involvement of inflammatory cytokines (including interleukins -1, -3, -6, -8, -11, and -18), and of nitric oxide (NO) has been shown in some studies [73, 74]; this phenomenon allowed to hypothesize that the increased production and release of proinflammatory cytokines, as well as endothelial dysfunction of central obesity, might influence megakaryocytopoiesis and circulating platelet volume [75].

The increased *in vivo* activation of circulating platelets is mirrored by the enhanced expression of activation-dependent adhesion molecules, and by increased plasma concentrations of sP-selectin; another index of *in vivo* platelet activation—that is, urinary excretion of 11-dehydro-TXB_2, the major enzymatic metabolite of TXA_2—is increased in women affected by visceral obesity, compared to non-obese women [76].

Relevant defects of platelet function in central obesity are related also to a reduced sensitivity to mediators—such as insulin, prostacyclin (PGI_2), and NO—which in lean subjects reduce platelet sensitivity to proaggregating stimuli [21].

Insulin, which physiologically reduces platelet responses to agonists both *in vitro* [77–79] and *in vivo* [77, 80, 81], mainly through a NO-dependent mechanism mediated by the increase of intraplatelet cyclic nucleotides $3',5'$ -cyclic guanosine monophosphate (cGMP) and $3',5'$ -cyclic adenosine monophosphate (cAMP) [78, 82], exhibits a deeply impaired antiaggregating effect in insulin resistant states, such as central obesity, type 2 diabetes mellitus with obesity and essential arterial hypertension [81, 83–85].

Furthermore, platelets from obese subjects or obese type 2 diabetic patients show defective responses to the NO/cyclic nucleotide/protein kinase pathway including the ability of NO and NO donors to increase cGMP, the ability of cGMP to reduce platelet calcium and consequently aggregation [86, 87]; similarly, also the ability of PGI_2 to increase cAMP and of cAMP to reduce platelet function are impaired in these patients [87].

As previously mentioned, a relevant factor causing plaletet dysfunction is the increased oxidative stress [40], which is present in central obesity, as a consequence of imbalance between ROS production and reduced levels of substances able to protect from the damage of free radicals and peroxides [88]. Several metabolic abnormalities of the patients with visceral obesity—that is, excess of circulating free fatty acids, of oxidized LDL, and of proinflammatory adipokines and cytokines—contribute to ROS production [88, 89].

Some adipokines and inflammatory cytokines such as TNF-α and leptin are involved in enhanced oxidative stress [88, 89]; furthermore, the increase in fat mass, as well as the pathological secretion pattern of adipocytes decrease the availability of antiinflammatory proteins such as adiponectin and ghrelin, which exert also protective effects against oxidative stress [15, 90, 91].

High concentrations of ROS influence platelet function by different mechanisms, including decreased NO bioavailability, calcium mobilization abnormalities and overexpression of membrane glycoproteins [40, 92–94].

Isoprostanes are a family of prostaglandin-like metabolites produced *in vivo* from esterified arachidonic acid of cell membrane phospholipids or lipoproteins [95, 96] through a ROS-dependent mechanism [96–99], completely independent of cyclo-oxygenase-1 (COX-1) activity [95–97]. Once released, they circulate in plasma and are available for receptorial interaction with platelets and cells of the vascular wall; in particular, 8-iso-prostaglandin-$F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), which is an abundant isoprostane compound formed *in vivo* in humans, induces vasoconstriction and amplifies the adhesive reactions and the aggregating responses of human platelets to agonists [100, 101].

Several studies showed a stimulatory interaction of isoprostanes with TXA_2 receptors [102, 103], which is prevented by TXA_2 receptor antagonism but not by COX-1 inhibition [99]. Recently, a further isoprostane binding site, responsible of cAMP reduction, has been recognized [103].

The increased levels of F2-isoprostanes observed in visceral obesity [104–107] can be involved both in the persistent platelet activation *in vivo* [104], and in the resistance to antiplatelet effects of aspirin [21].

4. Alterations of the Vascular Smooth Muscle Cells in Obesity

4.1. Role of Vascular Smooth Muscle Cells in Physiological Conditions. In physiological conditions VSMC play an essential role in providing structural integrity of the vessel wall and in controlling vascular tone and blood pressure [108, 109]; in particular, this cell type is the main target of the effects of endothelium-released NO, which stimulates the synthesis of cGMP, thus preventing the calcium release from intracellular stores [110, 111]. The inhibition of calcium-dependent Rho/Rho kinase pathway is a relevant mechanism involved in the modulation of VSMC relaxation induced by the NO/cGMP/PKG pathway [112].

The surface complex system which regulates VSMC responses and modulates the contractile process involves the expression of receptors for catecholamines, acetylcholine, serotonin, histamine, purinergic mediators, angiotensin II (Ang II), bradykinin, neuropeptide Y, Vasoactive Intestinal Polypeptide (VIP), vasopressin, oxytocin, prostanoids, leukotrienes, and growth factors [PDGF, Epidermal Growth Factor (EGF), TGF-β, Fibroblast Growth Factor (FGF), insulin, and Insulin-like Growth Factor (IGF-1)] [113, 114]. The signal transduction system following membrane activation is played by guanine nucleotide regulatory proteins, phosphoinositide metabolism, cyclic nucleotides (cAMP and cGMP), and calcium [113, 114].

Different agonists modulate VSMC responses by activating tyrosine kinases through receptor and nonreceptor mechanisms. In particular, IGF-1 through its specific receptor leads to direct activation of extracellular signal regulated kinases (ERK 1/2), whereas Ang II and other mediators activate tyrosine kinase pathway by indirect mechanisms such as increased hydrogen peroxyde (H_2O_2) production mediated by NADPH oxidase [113, 114].

Furthermore, VSMC which exhibit functional insulin receptors able to activate the classical signaling pathways—that is, Insulin Receptor Substrates/phosphatidylinositol 3-kinase (IRS/PI3-K) pathway and Mitogen-Activated Protein Kinase (MAPK) pathway [115–118], are targets of insulin action [115]. Through the PI3-K pathway, insulin stimulates glucose transport, induces the well-differentiated contractile state, antagonizes the effects of PDGF and increases NO production through NO synthase (NOS) activation [22, 78, 115, 116]; in the presence of PI3-K inhibitors, insulin, via the MAPK pathway, influences chemotaxis [117], DNA synthesis and proliferation [118–120].

In our previous studies in cultured human and rat arterial VSMC, we observed that insulin, through NOS activation with PI3-K-dependent mechanism, elicits a concentration-dependent increase of cGMP levels, increases cAMP content, and enhances the effects of the PGI_2 analogue Iloprost, of β -adrenoceptor agonists and of forskolin on cAMP levels

[22, 78, 84]. Insulin attenuates also the agonist-induced increase of intracellular calcium by inhibiting the inositol-triphosphate sensitive calcium release from intracellular stores and by some other biochemical mechanisms mediated by the PI3-K pathway [22, 78, 84, 115].

Furthermore, insulin can influence the hemodynamic balance also through the synthesis and secretion of vasoconstrictive agents such as ET-1 [22, 78, 84, 115].

Finally, via the cooperation of the two pathways, insulin activates the Hypoxia Inducible Factor (HIF)/Vascular Endothelial Growth Factor (VEGF) pathway [22, 78, 121, 122]. HIF-1 represents a "master switch" protein generated in response to hypoxia, able to influence erythropoiesis, vasomotion, glucose metabolism, cell proliferation and survival, iron metabolism and angiogenesis; in normoxic conditions, the HIF-1 system is also induced by cytokines and growth factors [123, 124]. VEGF is a mitogen and a survival factor for endothelial cells, able to induce vascular permeability and to regulate physiological and pathological angiogenesis, with a particular role in the postischemic revascularization [125].

4.2. Vascular Smooth Muscle Cells in the Pathogenesis of Atherosclerosis. Following repeated or chronic arterial wall injury, such as in arterial hypertension and exposure to other cardiovascular risk factors, VSMC respond by migration into the intima, secretion, as well as by increased proliferation [38, 126, 127].

VSMC migration to the intima from the media depends on mechanisms regulated by soluble growth factors/chemoattractants, as well as by interactions with extracellular matrix [38, 126, 127]. The secretion process increases extracellular matrix formation as well as the release of proteins involved both in the digestion of major components of the extracellular matrix, such as MMPs [128–130], and in angiogenesis, such as HIF-1 and VEGF [131–134].

A stimulating role on VSMC is exerted by cytokines and growth factors, including IL-1β, IL-6, TGF-β 1, TNF-α, thrombin, bFGF, IGF-1, PDGF, urokinase plasminogen activator (u-PA), Ang II, and VEGF [38, 126, 127, 133–137]; cytokine-dependent activation, in particular, increases the synthesis of MMPs and their processing from inactive zymogens to the active enzymes [128–130, 138–141]; the same effect has been recently described for C-reactive protein (CRP) [140], and for sCD40L, which increases VSMC proliferation and migration through the MMP-9 pathway [47, 142].

Enhanced proliferation of VSMC has been considered for many years only a mechanism involved in atherosclerotic plaque formation; however, since plaques prone to rupture (the so called "unstable" plaques) show a paucity of VSMC compared with the "stable" ones [143], it has been recently recognized a beneficial role of VSMC also in plaque stabilization and therefore in one of the main mechanisms involved in the prevention of cardiovascular events which are the consequence of plaque rupture and superimposed thrombosis [144]. In recent years, the role of VSMC apoptosis within the atherosclerotic plaques [145, 146], has been considered one of the major causes of plaque rupture by thinning the fibrous cap [147, 148]. Furthermore, VSMC apoptosis has proinflammatory effects and increases macrophage infiltration through the release of IL-1α and the up-regulation of MCP-1 and interleukin-8 (IL-8), responsible of macrophage infiltration *in vivo* [146, 148, 149]; *in vitro* studies showed that VSMC apoptosis also promotes both thrombin generation [150], and vascular calcification [151].

Finally, through the generation and release of microparticles, apoptotic vascular cells are thrombogenic locally, thus contributing to increase the thrombogenic potential of the lipid core [152], and systemically [153]; evidences from *in vitro* studies support this effects by showing that microparticles containing TF are released by cultured VSMC in response to stimuli mimicking minimal apoptosis or flow conditions [154].

Migration and proliferation of VSMC are also under the inhibitory control of the nuclear receptors Peroxisome Proliferator-activated Receptor (PPAR) alpha [155, 156] and gamma [157].

4.3. Impairment of Vascular Smooth Muscle Cells in Insulin Resistance States and Obesity. In obese subjects several studies showed an impaired arterial vasodilation, mainly involving cerebral, mesenteric, coronary, and skeletal muscle districts [158, 159]; a pivotal role in this phenomenon is played by endothelial dysfunction related to increased secretion of proinflammatory cytokines, reduced circulating levels of adiponectin, and enhanced release of free fatty acids: all these abnormalities alter gene expression and cell signaling in vascular endothelium, cause vascular insulin resistance, modify the release of endothelium-derived factors, and increase vascular oxidative stress [158–163]. In particular, the altered pattern of adipocytokine secretion characterizing central obesity—that is, reduced adiponectin and elevated levels of leptin, resistin, TNFα, and IL-6—increases the production of superoxide anion (O_2^-), that interferes with NO availability, thus reducing vasodilation [163, 164].

Furthermore, insulin resistance, which characterizes human obesity and involves also the vascular effects of the hormone [115, 123, 165, 166], can determine *per se* hemodynamic consequences by impairing the balance between the vasodilating insulin actions exterted via the NO/cGMP/PKG pathway and the vasoconstricting-ones mainly exerted via ET-1 in favour of the last-ones, as extensively reviewed [22, 78, 84, 115, 123, 167].

Since VSMC from animal models of insulin resistance show alterations in mechanisms involved in vasodilation, migration, and proliferation, a role of these vascular cells in hemodynamic alterations of obesity cannot be ruled out [22].

As previously mentioned NO/cGMP/PKG pathway plays a key role in VSMC-dependent vascular responses in physiological states [110, 111]: in particular, PKG initiates several phosphorylation events leading to VSMC relaxation through a reduction of free intracellular calcium levels, a decreased sensitivity of the contractile apparatus to calcium, a rearrangement of cytoskeleton, a dephosphorylation of myosin light chain, and a phosphorylation of two filament-actin

binding proteins, Vasodilatory-Stimulated Phosphoprotein (VASP) and the 20-kDa heat shock-related protein (HSP-20) [111]. PKG is also involved in VSMC proliferation and differentiation via its ability to modulate gene expression and protein synthesis [111].

Studies from our laboratory showed that VSMC from obese, insulin-resistant Zucker fa/fa rats—the classical animal model of insulin resistance due to defects in leptin receptors—show a reduced insulin ability to increase NO synthesis [121], and a reduced response to the NO/cGMP/PKG pathway [168]. In particular, VSMC from obese Zucker fa/fa rats shows: (i) baseline higher cGMP concentrations due to a reduced catabolism by phosphodiesterases; (ii) impairment of the NO ability to increase cGMP by activating the soluble guanylate cyclase; (iii) reduction of the NO and cGMP ability to activate PKG, as mirrored by a reduced ability to phosphorylate VASP at serine 239 and to activate phosphodiesterase 5 [168]. Interestingly, we also observed that VSMC from obese insulin-resistant Zucker fa/fa rats also show higher levels of O_2^-, and that antioxidants prevent the multiple defects of the NO/cGMP/PKG pathway, whereas H_2O_2 reproduces these defects in VSMC from lean, insulin sensitive Zucker fa/+ rats [168]: these data support the pivotal role of oxidative stress in the reduced response to NO in this animal model of obesity and insulin resistance, and can explain the reduced endothelium-independent relaxation observed by some authors in these animals *in vivo* [169].

Other investigations in animal models showed that increased proliferation and migration of VSMC are a main feature accounting for atherogenic arterial lesions in insulin resistant states [170, 171]. In a rat model of obesity and type 2 diabetes mellitus, it has been described both a numerical increase and functional abnormalities in intimal VSMC and the occurrence of VSMC accumulation in atherosclerotic lesions, with a direct correlation between VSMC proliferation and insulin concentrations [170, 172, 173]; the involvement of endogenous cytokines (especially TNF-α), and the receptors of advanced glycation end products (AGE) [RAGE] in neointimal formation in obese Zucker rats has been recently recognized [174].

These observations are clinically relevant for the insulin resistant patients who undergo revascularization procedures in coronary arteries and in other vascular districts [175–177], since VSMC migration and proliferation lead to excessive neointima formation, which is the primary mechanism responsible of restenosis [176]. It has also been observed that in patients with type 2 diabetes the compensatory hyperinsulinemia associated with insulin resistance strongly predicts neointimal VSMC proliferation [178], and that insulin resistance and endothelial dysfunction are independent predictors of early restenosis after coronary stenting in humans [179].

5. Influence of Adipocytokines on Platelets and Vascular Smooth Muscle Cells in Obesity

As previously mentioned, the altered pattern of adipocyte-derived hormones and cytokines is deeply involved in the chronic proinflammatory state characterizing patients with central obesity and partially accounts for their increased cardiovascular risk [5–7, 9–11, 15, 18].

Changes in adipocyte-derived factors enhance oxidative stress by activating oxidases, interfere with NO availability and influence cell proliferation and apoptosis [91, 171, 180, 181].

Several evidences indicated that adipokines and cytokines influence platelet production and responses and VSMC function. In this part of the paper we will focus on the effects of the different adipocytokines on these cells deeply involved in atherogenesis and atherothrombosis.

5.1. Interleukin-6 (IL-6). Interleukin-6 (IL-6) is a multifunctional proinflammatory cytokine produced by different cell types, including those present in adipose tissue [174, 182]: adipose tissue, in particular, contributes to up to 35% of circulating IL-6 levels [174].

In healthy individuals IL-6 expression is due to a tight regulation dependent on a complex hormonal network related to glucocorticoid and catecholamine secretion [182, 183].

Increased IL-6 expression and circulating levels have been associated with a variety of diseases, including metabolic and vascular diseases, such as central obesity, the metabolic syndrome, type 2 diabetes mellitus, atherosclerosis and, in particular, atherosclerotic coronary artery disease [182, 183].

IL-6—together with other cytokines—is a key risk factor for the development of atherothrombotic diseases due to its effect in plaque development and destabilization via release of other proinflammatory cytokines, oxidation of lipoproteins by phospholipases, stimulation of acute phase protein secretion, release of prothrombotic mediators, and activation of MMPs [183, 184]. Moreover, the increased ROS formation by vascular enzyme systems under proinflammatory conditions may play a critical role in the cross talk between IL-6 and other vasoactive substances, such as Ang II and catecholamines [185–187].

5.1.1. IL-6 and Platelets. As far as megakaryocytopoiesis is concerned, IL-6 acts synergistically with thrombopoietin (TPO), other interleukins, and growth factors in promoting the maturation of megakaryocyte precursors [188–192]; actually, *in vivo* administration of IL-6 to humans increases circulating platelet counts [190, 192]. This effect may explain the association between the increased markers of chronic inflammation and the elevated platelet count in obese women [193]: this phenomenon has been considered prothrombotic, since higher platelet counts are associated with adverse clinical outcomes in patients with acute coronary events [194].

As summarized in Figure 4, IL-6 is responsible of an acute prothrombotic state [184, 195] through mechanisms involving: (a) increased expression of TF, fibrinogen, factor VIII and vWF; (b) activation of endothelial cells; (c) reduced levels of hemostasis inhibitors, such as antithrombin and protein S [195]. Furthermore, IL-6 influences platelet function

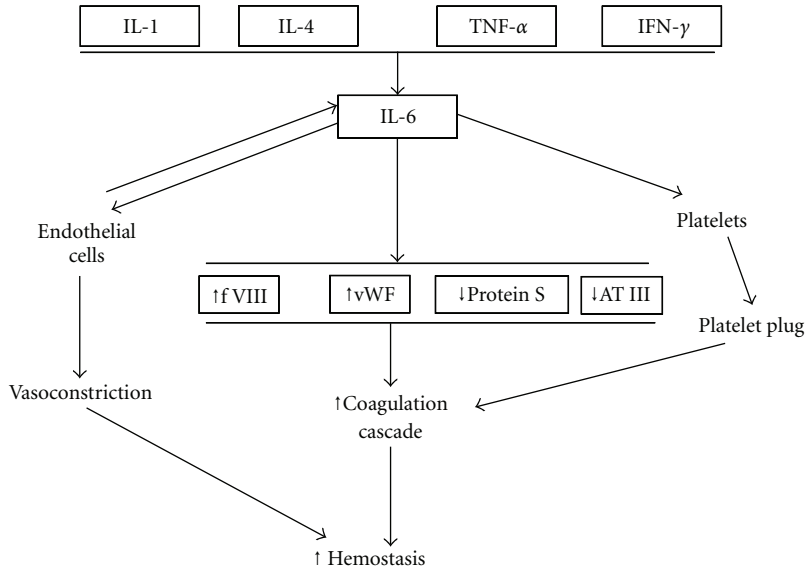

Figure 4: Mechanisms involved in the alterations of the coagulative balance induced by interleukin-6 (IL-6). IL-1, interleukin-1; IL-4, interleukin-4; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; f VIII, factor VIII, vWF, von Willebrand factor; AT III, antithrombin III.

by enhancing thrombin-induced activation [195], and modulates platelet responses by increasing ROS production [174].

5.1.2. IL-6 and VSMC. IL-6 influences both vessel wall cells and their progenitors; beyond its effects on resident endothelial cells, IL-6 exerts pro-angiogenic actions by stimulating migration and proliferation of circulating endothelial progenitors [196].

As far as VSMC are concerned, proinflammatory mediators increase in these cells synthesis and release of IL-6 [197]. Additionally, IL-6 exerts specific effects on VSMC: in particular, it is involved in growth factor-dependent VSMC migration, by interplaying with VEGF and TNF-α [198–200], and stimulates VSMC proliferation with PDGF-dependent and -independent mechanisms [201, 202].

These effects explain the clinical observation that increased IL-6 levels both in coronary and systemic circulation are a risk factor for restenosis after angioplasty [203, 204].

5.2. Tumor Necrosis Factor-α (TNF-α). TNF-α is a pleiotropic, proinflammatory cytokine, produced as 17 kDa protein and secreted as a 51 kDa trimer by a variety of cells including not only adipose tissue (see below) but also macrophages, natural killer cells, T-cells, endothelial cells and VSMC [205]; its presence has been also recognized in human atheroma [206].

Adipose tissue has been identified as one of the main sources of TNF-α: the majority of TNF-α produced in adipose tissue is derived from infiltrating macrophages and not from mature adipocytes [8, 13–15, 207].

Several studies evidenced an increased TNF-α circulating level both in obese nondiabetic subjects and in type 2 diabetic patients [13, 207–209] and hypothesized its involvement in the pathogenesis of obesity-linked insulin resistance [209–212].

As far as the TNF-α effects on vascular function are concerned, it has been observed that acute administration of TNF-α exerts *per se* a vasodilatory effect, but impairs endothelium-dependent vasodilation in response to insulin and acetylcholine in healthy humans [212–214]; furthermore, it inhibits the vasodilating actions of insulin in vessels of rat skeletal muscle [213–215]; the TNF-α interference with endothelial-mediated vasodilation is also due to shortening the half-life of eNOS mRNA in endothelial cells [215, 216] and to the increase of ET-1 synthesis and spillower [216].

TNF-α induces inflammatory changes in vessel wall by activating the transcription factor Nuclear factor-$\kappa\beta$ (NF-κ B) [217], which increases the expression of ICAM-1 and VCAM-1, and the production of MCP-1 and M-CSF from endothelial cells and VSMC.

Plasma TNF-α concentrations predict vascular damage, since they are associated with early atherosclerosis in middle-aged healthy men [218]; furthermore, elevations of TNF-α in the stable phase after myocardial infarction were associated with an increased risk of recurrent coronary events [219].

5.2.1. TNF-α and Platelets. *In vitro* studies showed that TNF-α promotes platelet aggregation [220] and ROS production mainly through activation of the arachidonic acid pathway [221, 222].

Other data indicate that TNF-α influences platelet function also by increasing the secretion of leptin, which acts as proaggregating hormone.

At present, the results from *in vivo* studies are not conclusive and there are not evidences to identify TNF-α as a prothrombotic or antithrombotic factor in human patients [223].

5.2.2. TNF-α and VSMC. Human VSMC are both source and target of TNF-α [205] which, together with interferon-γ and IL-1, stimulates IL-6 production by this cell type [224]. Through NF-κ B pathway, TNF-α increases synthesis/release of MMP from VSMC [217]. Thus, TNF-α is deeply involved in plaque inflammation and instabilization also by the mechanisms involving VSMC as described in the first part of this review.

5.3. Leptin. Leptin is a 167-amino acid adipokine, primarily synthesized and released by mature adipocytes, although expressed also in many other tissues including muscle, placenta and gastric epithelium. Its circulating levels are highly correlated with BMI [225].

Leptin receptors have been identified both in the hypothalamus and in extrahypothalamic tissues and its main role is to inform the brain regarding the amount of stored fat, thus primarily regulating food intake and energy expenditure [226]; however, in obese humans increased leptin levels are unable to induce weight loss: this phenomenon is attributed to a selective resistance to its metabolic actions [227].

Leptin, which has a structural and functional relation to proinflammatory cytokines such as IL-6, also influences angiogenesis, inflammation, arterial blood pressure and secretion of other adipokines [10, 180, 181, 207].

In animal models, chronic hyperleptinemia is involved in oxidative stress by decreasing plasma levels of the antioxidant enzyme paraoxonase-1, an activity linked to circulating lipoproteins [228]. This leptin effect is followed by increased plasma and urinary concentration of isoprostanes reflecting an increased oxidative stress [228]. Evidences for an involvement of leptin in atherosclerosis have been recently provided by direct leptin administration in apolipoprotein deficient mice [229] and by the finding that ob/ob mice which lacked functioning leptin gene are resistant to atherosclerosis despite the presence of obesity and diabetes [230]. Also in humans, the vascular actions of leptin are considered proatherogenic and the increase of its circulating levels due to adiposity has been involved in the pathogenesis of vascular damage [231].

At present, clinical investigations considered leptin as an independent risk factor for cardiovascular [232–234] and cerebrovascular diseases [235, 236], evidencing that its plasma concentrations are independently associated with the intima-media thickness of the common carotid artery [237], and with the degree of coronary artery calcification in patients with type 2 diabetes mellitus, after controlling for adiposity and CRP [238, 239]; furthermore, hyperleptinemia could be involved in the increased risk of postangioplasty restenosis [179, 240].

5.3.1. Leptin and Platelets. The pro-thrombotic actions of leptin *in vivo* are related to an influence on platelet function, and on coagulation/fibrinolysis balance, resulting in enhanced agonist-induced platelet aggregation and increased stability of arterial thrombi [241–243].

The long form of the leptin receptor (Ob-Rb) is present in human platelets and can be related to platelet activation by a specific pathway downstream of leptin-induced Janus kinase 2 (JAK2) activation including PI3-K and phospholipases $C\gamma_2$ and A_2, which influence cAMP hydrolysis, GPIIb/IIIa expression, and thromboxane synthesis [241]: these findings induced to consider circulating plaletets as a major target of leptin action, suggesting a possible direct link between obesity and thrombotic complication [241, 242].

Studies *in vitro* showed that leptin synergizes with subthreshold concentrations of agonists—such as ADP and thrombin—to induce platelet aggregation [242–245], but is unable to directly aggregate platelets. The involvement of leptin in the increased platelet activation in human obesity is not universally accepted, since recent studies provided conflicting results about platelet responsiveness to leptin in overweight and obesity [246, 247]; actually, in one study the effect of leptin on ADP-induced platelet aggregation is attenuated in obese individuals due to the receptor desensitization [245], whereas different results have been provided in another report [247].

Normal weight subjects undergoing complete caloric deprivation have an increased sensitivity of hemostatic responses to leptin [248]: this phenomenon indicates that the sensitivity of leptin receptors on platelet membranes is influenced by body composition [248]. In light of this, the resistance to leptin in overweight or obese patients could represent a protective mechanism against the excess pro-thrombotic stimulation produced by obesity-related hyperleptinemia [249].

However, other mechanisms by which leptin may contribute to vascular damage—such as inflammation, oxidative stress, endothelial dysfunction, as well as increased sympathetic tone—are preserved in obese subjects [240, 250–252] and can contribute to pro-thrombotic action of leptin [240, 253].

5.3.2. Leptin and VSMC. Leptin exerts hemodynamic actions, even though its effects on the arterial wall have not been fully elucidated at present. Evidences in animals and humans showed that leptin administration induces acute vasodilation in different vascular districts, including human coronary arteries [207, 254, 255].

The involvement of a receptor-mediated NO release from endothelium has been shown in several, but not all, experimental models of leptin-induced vasodilation [255, 256]; despite the increase of NO release, acute leptin administration induced blood pressure reduction only in sympathectomized animals, likely for a compensatory activation of the sympathetic nervous system [256, 257]. Insulin interacts with leptin to modulate vascular responses, mainly by enhancing NO production by endothelium [258].

Due to the presence of active receptors in VSMC [259], leptin induces VSMC migration, proliferation, and expression of MMP-2, as shown in human aorta *in vitro* [260]; furthermore, *in vitro* studies showed that leptin stimulates osteoblastic differentiation of VSMC and hydroxyapatite production, thus explaining the relationships between circulating levels of leptin and the degree of coronary artery calcification [238, 239, 261].

Indirect mechanisms responsible of other leptin effects on VSMC are referred to oxidative stress which can cause endothelial or VSMC damage and stimulation of low-grade vascular inflammation [262].

5.4. Adiponectin. Adiponectin is a 30-kDa protein collagen-like molecule that shares substantial homology with subunits of complement factor C1q [207, 263, 264]. It is expressed almost exclusively in mature adipocytes where is the most abundant adipokine synthesized and released locally and in the blood stream. It accounts for 0.01% of the total plasma proteins in form of trimer, hexamer and high molecular weight 12-to 18-mer [207, 263, 264]. Adiponectin synthesis is detectable also in human and murine cardiomyocytes [265].

As opposite to the other adipokines, circulating adiponectin is negatively related to the increase of fat mass likely owing to the abnormal hormonal milieu mainly caused by the inhibitory effects exerted by the increased local TNF-α levels, by the oxidative stress and by the proinflammatory state which prevail in central obesity [207, 264, 266, 267]; its secretion is restored as a consequence of weight loss [267].

Adiponectin exerts insulin-sensitizing effects by increasing glucose uptake, NO production, and free fatty acid oxidation [268–270] and shows an antiinflammatory activity mainly through a cAMP-mediated interference with NF-κ B signaling [271].

In vascular wall, the antiinflammatory properties of adiponectin—which account for its antiatherogenic effects—reduce cell expression of adhesion molecules and scavenger receptors [272, 273]. This phenomenon is mediated by the inhibition of the effects of TNF-α and Ang II on both endothelial cells (expression of adhesion molecules, protection, increase of permeability, production of ROS) and macrophages (decrease of cytokine production mediated by NF-κ B signaling) [268, 271, 272].

The vasculo-protective effects of adiponectin have been recently confirmed in clinical studies, by showing that its decreased levels contribute to the metabolic and vascular abnormalities in obese subjects [264, 268, 274].

5.4.1. Adiponectin and Platelets. In animals adiponectin plays a role as antithrombotic factor [275]. Actually, there is an accelerated thrombus formation after carotid arterial injury in adiponectin knockout mice in comparison to wild-type ones: the potential involvement of platelets in this effect is suggested by the presence of active adiponectin receptors AdipoR1 and AdipoR2 in wild-type mice and by the enhanced platelet response to ADP and collagen in adiponectin knockout animals [275]. The same receptors are present in isolated human platelets and in human megakaryocytic cell lines; in humans, however, adiponectin does not influence platelet activation by ADP and collagen [276].

It has been hypothesized that adiponectin exerts indirect anti-thrombotic effects by decreasing the circulating concentrations of TNF-α and IL-6 and by interfering with their pro-thrombotic activities, mainly dependent on increased oxidative stress and decreased NO bioavailability [20, 277, 278].

5.4.2. Adiponectin and VSMC. Adiponectin suppresses proliferation and migration of smooth muscle cells by directly binding to several growth factors, particularly PDGF BB, FGF, and heparin-binding epidermal growth factor-like growth factor (HB-EGF) [279, 280].

Following experimental vascular injury, immunohistochemical analysis shows the presence of adiponectin in the walls of the catheter-injured vessels but not in intact vascular walls [281]; thus, it can be hypothesized that adiponectin secreted from adipose tissue reaches the injured arteries after the lesion of the endothelial barrier and accumulates in vascular walls, thus reducing the atherogenic process [281].

Finally, adiponectin may favor plaque stabilization decreasing MMP activity by modulating the expression of the Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) through increased IL-10 secretion [282].

5.5. Ghrelin. Ghrelin is a 28-amino acid peptide hormone, predominantly produced by the stomach [283], which acts as a natural ligand of the Growth Hormone (GH) secretagogue (GHS) receptor type 1a (GHS-R1a), and plays a role in the central control of appetite together with leptin [284]. Circulating ghrelin levels are inversely related with degree of obesity evaluated by BMI [285].

More recently, it has been demonstrated that ghrelin also influences the cardiovascular system, by decreasing sympathetic activity and producing vasodilation through an endothelium-independent mechanism [286].

Furthermore, ghrelin exerts antiinflammatory effects in vessels, improves left ventricular function and shows antiapoptotic actions on cardiomyocytes [286].

It has been suggested that the decrease of ghrelin in obese patients is involved in their enhanced cardiovascular risk and that ghrelin administration exerts protective effects in patients with obesity-related metabolic syndrome [287].

5.5.1. Ghrelin and Platelets. The protective cardiovascular effects of ghrelin likely do not involve platelets, since *in vitro* this hormone does not affect platelet aggregation or adhesion [276].

5.5.2. Ghrelin and VSMC. VSMC are targets of ghrelin, which inhibits *in vitro* Ang II-induced proliferation and contraction in a dose-response manner via the cAMP/PKA pathway [288], and prevents vascular calcification in rats [289].

5.6. Apelin. Apelin is an endogenous peptide ligand for the orphan G-coupled APJ receptor, which exhibits close homology with the angiotensin-like 1 receptor [290]. It exists at least in three molecular forms, consisting of 13, 17, or 36 amino acids, all originating from a common 77-amino acid precursor, and is present in several tissues, primarily in the vascular endothelium [290]. Recently, apelin has been identified in adipose tissue [291], where its gene expression

is increased by insulin, steroids and TNF-α [292, 293]. Its synthesis and release are up-regulated in obesity, whereas its expression in adipose tissue and its circulating levels are reduced by weight loss [292].

Apelin is mainly considered a vascular hormone: actually, it induces an endothelium-dependent vasodilation [294, 295], exerts inotropic effects [296], and plays a protective role in the pathogenesis of heart failure by modulating the harmful effects of Ang II [294, 295].

Apelin is down-regulated in patients with heart failure and up-regulated by a favourable left ventricular remodeling [295].

The role of apelin-APJ system in atherogenesis is controversial. Some apelin actions—including modulation of endothelial oxidative stress and macrophage activation, NO-dependent vasorelaxation and reduction of arterial blood pressure [295, 297, 298]—are clearly protective, whereas other effects on arterial vessels might be detrimental by favouring the atherogenic damage [299].

5.6.1. Apelin and VSMC. In the vascular system, the apelin-APJ system is expressed in both endothelium—where it is mitogenic [300]—and in VSMC [301]. Acute apelin administration in man causes NO-mediated arteriolar vasodilation [294]. At present, data about the direct effects of apelin on VSMC are incomplete; it has been suggested an influence on myosin light chain phosphorylation via APJ receptors, responsible of vasoconstriction [302], and a proliferative effect on VSMC, induced by oxidative stress [299, 303].

5.7. Visfatin. Visfatin, previously recognized as a protein involved in immune B-cell maturation [pre-B cell colony-enhancing factor (PBEF)] [304], is abundantly expressed in visceral adipose tissue and is up-regulated in some, but not in all, the animal models of obesity [305]. Preliminary studies suggest that plasma visfatin concentrations are increased in humans affected by abdominal obesity and type 2 diabetes mellitus [306, 307].

Although it is not a cytokine, visfatin expression is up-regulated by cytokines: several studies showed its involvement in metabolic and vascular homeostasis [308, 309]. Metabolic insulinomimetic effects, characterized by reduction of hepatic glucose production and stimulation of peripheral glucose utilization, has been attributed to binding to insulin receptor at a site different from that of insulin [308], even though recent results excluded a visfatin-dependent direct activation of insulin signaling pathway and attributed its action to nicotinamide phosphoribosyltransferase activity (Nampt) [310]. Vascular effects are both chronic and acute (see Figure 5): chronic exposure to high visfatin concentrations—such as in obesity and in type 2 diabetes mellitus—promotes endothelial dysfunction, angiogenesis and atherosclerotic plaque instabilization, whereas acute visfatin administration stimulates eNOS expression and activity in endothelial cells [309] and directly protects cardiomyocytes against the detrimental effects of acute ischemia-reperfusion injury [311].

5.7.1. Visfatin and VSMC. Visfatin influences VSMC phenotype maturation from a proliferative noncontractile to a non-proliferative contractile one required for vasomotor function [312], and promotes VSMC proliferation in perivascular adipose tissue by a paracrine mechanism [313].

5.8. Resistin. Resistin has been originally identified as an adipocyte-secreted peptide able to induce insulin resistance in rodents; in humans it is represented by a 12.5 kDa cysteine-rich protein of 108 amino acids [314]. Also in humans resistin is produced by adipose tissue and may act both in paracrine and in endocrine fashion [314]: however, in contrast to mice, only a low level of expression of resistin has been found in mature adipocytes in humans [315, 316]. Furthermore, resistin expression has been demonstrated in bone marrow, trophoblastic cells of placenta, pancreas, synovial tissue, and circulating blood cells [317].

Resistin is an important regulator of glucose homeostasis, adipogenesis, and, potentially, inflammation [317]; in particular, it can induce insulin resistance by regulating adipose tissue deposition through a negative feedback mechanism [317], and exerts proinflammatory effects through activation of the transcription factor NF-κ B [318].

The interplay between resistin and vascular wall cells can potentially contribute to the development of atherosclerotic lesions (Figure 6): in particular, it favors angiogenesis by inducing endothelial cell growth activation and migration, mainly by increasing ET-1 release [319, 320], and potentiating the effect of CD40L [321]; furthermore, it is involved in lipid storage in macrophages [317, 322].

5.8.1. Resistin and VSMC. Resistin induces proliferation of cultured human aortic VSMC through both ERK 1/2 and Akt signaling pathways [323]. Furthermore, hypoxia increases resistin expression in cultured rat VSMC [323].

5.9. Endothelin. Increased circulating levels of ET-1 have been observed in patients affected by central obesity and metabolic syndrome [324]. ET-1 elevation is proportional to hyperinsulinemia [324, 325], and weight loss by diet intervention reduces both serum insulin and ET-1 [326].

The increase of ET-1 accounts for a prevailing vasoconstrictive effect of insulin in insulin resistant states, in which the insulin-induced, PI3-K-mediated increase of NO is impaired [325]; furthermore, ET-1 contributes *per se* to vasoconstriction by influencing calcium fluxes, by activating the renin-angiotensin system, and by inducing VSMC hypertrophy [327].

5.9.1. ET-1 and Platelets. Platelets are a potential target of circulating ET-1. However, as recently reviewed, ET-1 effects on platelets are still conflicting [328]: some studies showed that *in vitro* exposure to ET-1 induces platelet activation or increases platelet responses to aggregating agonists [327–329], other studies, however, failed to detect any direct effect [329] or even showed a decrease in platelet responses [328].

These conflicting results may be due to complex interactions between platelet ET(A) and ET(B) receptors.

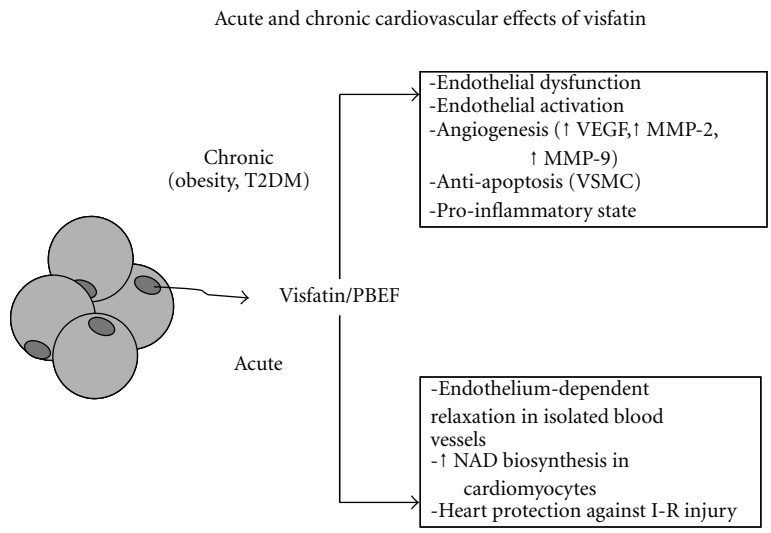

Figure 5: Acute and chronic cardiovascular effects of visfatin. VEGF, vascular endothelial growth factor; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; PBEF, pre-B cell colony-enhancing factor I-R, ischemia-reperfusion.

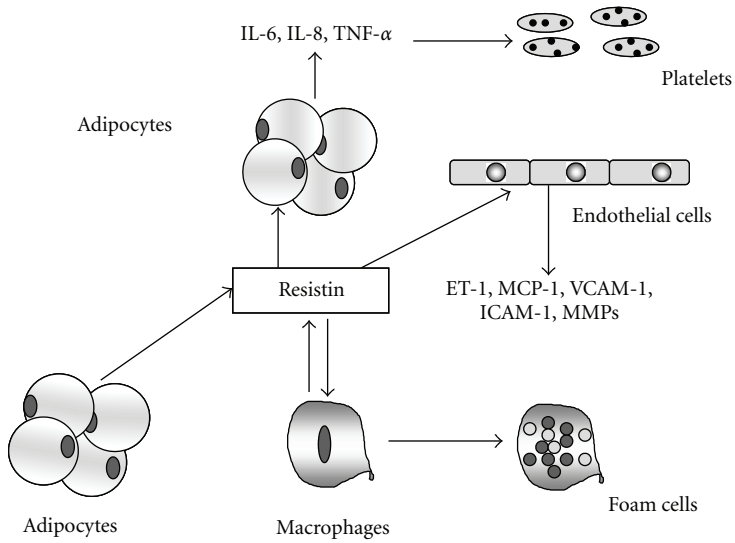

Figure 6: Potential vascular effects of resistin. IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrosis factor-α; ET-1, endothelin-1; MCP-1, monocyte chemoattractant protein-1; VCAM-1, Vascular Cell Adhesion Molecule-1; ICAM-1, Intercellular Adhesion Molecule-1; MMPs, matrix metalloproteinases.

5.9.2. ET-1 and VSMC. VSMC are both a source and a target of ET-1 [330–333]. As recently reviewed [333], ET-1 stimulates VSMC proliferation [334], migration [335], contraction [336], extracellular matrix synthesis and remodeling [337, 338], and expression of other proatherogenic growth factors such as PDGF and TGF-β [339].

6. Conclusions

The major adverse consequences of central obesity are related to the development of type 2 diabetes mellitus and of atherothrombotic vascular diseases, which account for a high disease-related mortality [1, 5–7]. As extensively confirmed, the main alterations of central obesity involved in vascular damage are recognized in the impaired systemic metabolic homeostasis as well as in the presence of an active low-grade chronic inflammatory process in tissues that are relevant for metabolism, such as adipose tissue, liver, muscle and arterial wall [6, 11, 13, 15, 19, 340].

In particular, the activation of an inflammatory process by adipose tissue is related to impairment of the secretion pattern of adipocytokines, including increased local availability of major cytokines [6, 7, 13, 14, 18, 340, 341], as well as enhanced synthesis and secretion of proinflammatory adipokines [13–15] and reduced availability of protective, insulin sensitizing peptides, including adiponectin and ghrelin [2, 15, 340–343].

On the basis of the large number of reviewed studies, a relevant role can be recognized to impairment of platelet and VSMC functions in the pro-thrombotic tendency, proinflammatory state and accelerated atherogenesis of the patients with central obesity [20–22].

Impaired synthesis and/or secretion of single adipocytokines which can interplay with platelets and VSMC are deeply involved in these phenomena.

As extensively reviewed, a large body of evidences showed detrimental actions of the increased synthesis and secretion of several mediators which act through pro-thrombotic and proliferative actions and oxidative stress, including TNF-α, interleukins and likely leptin [342]. However, also the lack of the protective effects of adipokines such as adiponectin and ghrelin has to be considered another relevant feature of the proatherogenic milieu characterizing the altered endocrine pattern of the patients with central obesity. In particular, adiponectin is a relevant anti-thrombotic adipokine, as widely reviewed [266, 268, 343] and emerging evidences underline ghrelin protective effects on insulin resistance, cardiovascular system, oxidative stress, and, likely, hemostatic balance [20, 104, 105, 343]: therefore, the reduced levels of this peptide in central obesity may be another deterimental feature increasing cardiovascular risk in obese subjects.

In summary, available evidences allow to hypothesize the presence of a complex scenario related to increased atherogenic and atherothrombotic risk in central obesity: in this context an impaired pattern of adipocytokine synthesis and secretion, rather than alteration of single mediators, has to be considered a major mechanistic link.

Review Strategy and Selection Criteria

Searches for original articles and reviews from 1985 to February 2010 focusing on obesity, hemostasis, vascular function and adipokines were performed in MEDLINE and PubMed electronic databases. The search terms were: "adipocytokines", "adipokines", "adiponectin", "apelin", "cytokines", "central obesity", "endothelin-1", "ghrelin", "insulin resistance", "interleukin-6", "leptin", "metabolic syndrome", "obesity", "overweight", "platelets", "platelet dysfunction", "resistin", "thrombosis", "Tumor necrosis factor-α", "vascular smooth muscle cells", and "visfatin". All papers identified were English-language, full-text papers and were selected on the basis of relevance and novelty; a priority was given to those published in peer-reviewed journals.

Abbreviations

ADP: Adenosine 5-diphosphate
AGE: Advanced glycation end products
Akt/PKB: Akt/protein kinase B
Ang II: Angiotensin II
BMI: Body mass index
cAMP: 3′, 5′ -cyclic adenosine monophosphate
CD40L: CD40 ligand
cGMP: 3',5'-cyclic guanosine monophosphate
COX-1: Cyclo-oxygenase-1
CRP: C-reactive protein
DAG: Diacylcyglycerol
EGF: Epidermal Growth Factor
eNOS: Endothelial NO synthase
ERK 1/2: Extracellular signal regulated kinases
ET-1: Endothelin-1
FACoA: Fatty acid coenzyme A
FFA: Free fatty acids
FGF: Fibroblast Growth Factor
H_2O_2: Hydrogen peroxide
HSP-20: 20-kDa heat shock-related protein
HSP-90: Heat shock protein-90
HIF-1: Hypoxia Inducible Factor-1
ICAM-1: Intercellular Adhesion Molecule-1
IGF-1: Insulin-like Growth Factor-1
IL-1:β Interleukin-1β
IL-6: Interleukin-6
IP3: Inositol, 1,4,5-triphosphate
IRS: Insulin Receptor Substrates
8-iso-$PGF_{2\alpha}$: 8-iso-prostaglandin-$F_{2\alpha}$
LDL: Low density lipoproteins
MAPK: Mitogen-Activated Protein Kinase
MCP-1: Monocyte Chemoattractant Protein-1
MIF: Macrophage migration inhibitory factor
MMPs: Matrix metalloproteinases
MPV: Mean platelet volume
NF-Bκ: Nuclear Factor-κ B
NO: Nitric oxide
O_2^-: Superoxide anion
PAF: Platelet activating factor
PAI-1: Plasminogen Activator Inhibitor-1
PBEF: Pre-B cell colony-enhancing factor
PDGF: Platelet-derived growth factor
PF-4: Platelet factor-4
PGI: Prostacyclin
PI3-K: Phosphatidylinositol 3-kinase
PIP: Phosphatidylinositol 4,5-bisphosphate
PKC: Protein kinase C
PKG: cGMP-dependent protein kinase
PPAR: Peroxisome proliferator-activated receptor
RAGE: Receptors of advanced glycation end products (AGE)
RANTES: Regulated on activation, normal T-cell expressed and secreted
ROS: Reactive oxygen species
sGC: Soluble guanylate cyclase
TF: Tissue factor
TGF-β : Transforming growth factor-β
TIMP-1: Tissue Inhibitor of Metalloproteinase-1
TNF-α: Tumor necrosis factor-α
TPO: Thrombopoietin
TXA: Thromboxane A_2
u-PA: Urokinase plasminogen activator
VASP: Vasodilatory-Stimulated Phosphoprotein
VCAM-1: Vascular Cell Adhesion Molecule-1
VEGF: Vascular Endothelial Growth Factor
VIP: Vasoactive Intestinal Polypeptide
VSMC: Vascular smooth muscle cells
vWF: Von Willebrand factor.

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Review Article

The Role of Adipose Tissue and Adipokines in Obesity-Related Inflammatory Diseases

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Obesity is an energy-rich condition associated with overnutrition, which impairs systemic metabolic homeostasis and elicits stress. It also activates an inflammatory process in metabolically active sites, such as white adipose tissue, liver, and immune cells. As consequence, increased circulating levels of proinflammatory cytokines, hormone-like molecules, and other inflammatory markers are induced. This determines a chronic active inflammatory condition, associated with the development of the obesity-related inflammatory diseases. This paper describes the role of adipose tissue and the biological effects of many adipokines in these diseases.

1. Introduction

Obesity may be considered as the result of a positive energy balance in conditions of energy excess. Correlated with economic, social, and lifestyle changes, it represents a common condition of different populations living in environments characterised by abundant calorie-rich food and low physical activity [1]. Hence, obesity is rapidly arriving at epidemic proportions in many parts of world and is becoming one of the major public health problems. More than 1 billion individuals are overweight and more than 300 million worldwide subjects can be classified as obese (with body mass index (BMI) of 30 kg/m^2 or higher) [1]. More than two thirds of American population is overweight, a common condition of other Western populations [2]. In particular, the highest frequency of obesity is observed in the United States, Europe and the Middle East and the lowest in sub-Saharan Africa and East Asia [3]. In these parts of world, this condition is very alarming, because it occurs in children and adolescents [4, 5]. Hence, the current obesity may be only considered the "tip of the iceberg", which will see young subjects develop the typical age-related diseases, because obesity predisposes to a variety of age-related inflammatory diseases, including insulin resistance (IR), type 2 diabetes, atherosclerosis and its complications, fatty liver diseases, osteoarthritis, rheumatoid arthritis, and cancer [6]. The obesity state is, indeed, characterized by what has been called "low-grade systemic inflammation", induced by different inflammatory mediators, as demonstrated for the first time by Hotamisligil et al. in 1993 [7].

The growing evidence on the obesity and the associated pathologies has led to understand the role of adipose tissue as an active potential participant in controlling the physiological and pathological processes. To date, the adipose tissue is considered as an endocrine organ able to mediate biological effects on metabolism and inflammation, contributing to the maintenance of energy homeostasis and, probably, pathogenesis of obesity-related metabolic and inflammatory complications [8].

This review describes the role of adipose tissue and evidences the biological effects and clinical significance of many adipokines in obesity-related inflammatory diseases.

1.1. Adipose Tissue: Heterogeneity and Functions. Adipose tissue is vital for the life of mammals. It represents the major source of fatty acids (FFA) in the postprandial fasting state for energy use and heat production [9]. Two types of adipose

tissue are present in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT) [9]. They have not only different functions, but also a different cellular composition and localization [9].

WAT constitutes the major component of body's adipose tissue, provides most of the total body fat and is the source of FFA, used as energy substrates for the generation through oxidative phosphorylation of adenosine triphosphate (ATP) high-energy bonds [9]. WAT is dispersed in different anatomic body's sites. Its major depots are intraabdominal around the omentum, intestines and perirenal areas, and subcutaneous in the buttocks, thighs and abdomen [9]. Hence, it is possible to identify several local WAT subgroups, including visceral, muscle, epicardial, perivascular and kidney. Furthermore, WAT seems to have two key functions, as recently demonstrated by Worzniak et al. and Juge-Aubry et al. [8, 10]. It is involved in the control of the metabolism through energy homeostasis, adipocyte differentiation, and insulin sensitivity. Besides, it affects inflammation, through a control mechanism mediated by antiinflammatory molecules and the activation of anti-inflammatory metabolic and immune pathways [8, 10]. In addition, each local WAT subgroups have specialised roles.

Its excessive accumulation in these body's sites might arise and determine the development of obesity and the obesity-related diseases. Much common is the WAT excess in the upper parts of body, the so-called "android obesity" or "central obesity", which represents a strong risk factor for some inflammatory pathologies [11]. The WAT excess in other lower body's sites gives rise to the so-called "gynoid obesity" with no metabolic complications [9, 11].

To understand the different WAT distribution and its different link with metabolic and inflammatory complications, several theories have been advanced. Among these, two major theories, not mutually exclusive, have been considered. The first is based on the anatomy of central obesity and its capacity to drain FFA and inflammatory mediators into the portal circulation, where they can act preferentially on the liver to affect metabolism [9]. The second considers cell biology and different properties of WAT cells linked with a major or minor risk to develop metabolic and inflammatory diseases [12]. Significant differences in expression of several genes between the different body's deposits of WAT in both rodents and human have been detected [13–15]. Interestingly, a different mediator profile has also been observed between visceral and peripheral WAT. This should seem to clarify the link between central obesity and metabolic complications. Besides, it also evidences the heterogeneity in nature and kind of WAT cells [9]. Several types of cells constitute WAT: mature adipocytes and a variety of other cells (i.e., preadipocytes, fibroblasts, endothelial cells, and macrophages), usually grouped and described as the "stroma vascular fraction" [9, 11, 16]. The adipocytes, preadipocytes, and macrophages have metabolic and inflammatory functions, which render WAT able to release several mediators with different biological effects in the WAT itself or other tissues, acting in paracrine or endocrine way [8, 10, 16–20]. In particular, the macrophages are responsible for the circulating levels of specific inflammatory molecules, determining the "low-grade" chronic obesity-related inflammation [8, 11, 16, 17, 21].

Unlike WAT, BAT provides energy expenditure from nonoxidative phosphorylation in form of heat largely for cold adaptation [22]. The uncoupling of phosphorylation in BAT is due to the activity of uncoupling protein-1, expressed on the internal mitochondrial membrane, which by creating a proton leak exhausts the electrochemical gradient needed for oxidative phosphorylation. As consequence, BAT affects energy use by producing heat from uncoupled oxidative phosphorylation [22]. Unlike WAT, BAT also presents a smaller number of fat cells, which have richer vascular supplies with more abundant mitochondrial chromogens, responsible for the brown colour [22]. BAT with a richer vascular supply responds more rapidly to sympathetic nervous system (SNS) stimulation, which then elicits heat production, rather than ATP production, from nonshivering cold adaptive thermogenesis [22]. Hence, BAT shows a different function respect to WAT, and, in most mammals, it is responsible of the heat for fever, the arousal state from hibernation and cold-induced-thermogenesis [22]. In humans, BAT is difficult to find postnatally [22]. However, the positron emission tomography has clearly shown in adult humans metabolically active BAT depots in cervical, supraclavicular, axillary and paraventral body's regions. These depots can be induced in response to cold and SNS activation [22–24]. This highlights BAT as a potential relevant target for both pharmacological and gene expression manipulation to combat human obesity [23, 24].

1.2. Fat Remodelling and Regulation of Energy Homeostasis. To understand the potential mechanisms involved in energy-rich condition under overnutrition, it is necessary to known the processes of energy homeostasis. Adequate body fat and energy homeostasis are ensured through a dynamic process of fat remodelling, without excessive weight gain or loss [22]. Conditions of increased appetite and food intake determine a positive energy balance with weight gain [22]. In contrast, satiety limits food consumption determining a negative energy balance and weight loss [22]. This process is mediated through hypothalamic neuropeptide regulation of appetite and satiety [22]. Energy expenditure is regulated by central and autonomic nervous systems, which achieve a balanced energy homeostasis depending on physiological needs [25]. Hence, conditions of more energy expenditure determine WAT lipolysis and the consequent augment of FFA, or, conversely, less energy expenditure states allow an increased fat storage [22]. This is controlled by parasympathetic nervous system (PNS) and SNS. PNS facilitates the fat storage and decreases the peripheral energy use [26]. SNS stimulates lipolysis, by increasing the release of FFA for increased energy expenditure [27]. Both WAT and BAT have SNS innervations and $\beta 3$ adrenergic receptors. BAT responds to cold-induced SNS activity with increased production of heat from uncoupled oxidative phosphorylation [27, 28]. WAT stimulated by upregulated SNS activity in response to cold increases the thermogenesis from oxidative phosphorylation

of FFA within liver, muscle and fat cells, which is increased in obesity [27].

Thus, energy homeostasis is achieved by balancing food intake with energy use. Conditions of increased calorie intake associated with decreased energy use induce obesity. In obese conditions, BAT mass and function are strongly decreased [22]. Obese individuals who are heat intolerant are less able to dissipate heat from both WAT and BAT [22].

2. Obesity and Inflammation: Causes, Mechanisms and Consequences

A regulated interaction between metabolic and immunity system exists [29]. Both over and undernutrition conditions influence metabolism and immune functions [30, 31]. Malnutrition conditions can suppress immune system and increase susceptibility to infections [30, 31]. Obesity, an energy-rich condition associated with overnutrition, impairs systemic metabolic homeostasis and elicits stress [29]. Stress has been especially linked to development of visceral obesity [29]. An inflammatory process is simultaneously activated by increased WAT mass in metabolically active sites, such as WAT itself, liver and immune cells [11, 16–18, 20]. This response determines a strong increase in circulating levels of proinflammatory cytokines, hormone-like molecules and other inflammatory markers, collectively defined "adipokines" [8, 18, 19]. To counteract the obesity-related stress, hypothalamic-pituitary-adrenal axis and central and peripheral components of autonomic nervous system are activated [32]. Under stress conditions they induce physiological responses. Chronic obesity-related stress induces a prolongation of these adaptive responses. This leads to an increased level of glucocorticoid, a steroid hormone able also to induce the development and differentiation of preadipocytes, favouring consequently the further increase of WAT mass [33]. On the other hand, the secretion of pro-inflammatory cytokines by WAT may act as additional chronic stimulus for activation of hypothalamic-pituitary-adrenal axis. Hence, a vicious cycle between metabolic and immune responses in obesity state is promoted, inducing a chronic active inflammatory condition able to determine the onset of obesity-related pathologies [29, 31].

The causes and mechanisms involved in obesity-induced inflammatory state are not fully understood, even if the link between inflammation and obesity has been indicated by epidemiological studies from 1950s onwards. The discovery of the production of proinflammatory cytokines in the WAT, such as tumour necrosis factor (TNF)-α, and TNF-α capacity to regulate insulin action has systemically also represented a driving force in this field [7].

Today, current opinion proposes that, under normal WAT conditions, adipocytes store lipids and regulate metabolic homeostasis, and resident tissue macrophages, with polarization essentially of M2 type, release anti-inflammatory cytokines [21]. M2 macrophages produce arginase (enzyme involved in the inhibition of nitric oxide synthase, iNOS) and IL-10, IL-1Ra anti-inflammatory cytokines [21, 34, 35]. In contrast, M1 type macrophages have a specific surface marker (CD11c+) and release iNOS and classical proinflammatory cytokines [21, 34, 35]. Normal WAT is, so, characterised by an anti-inflammatory tissue milieu able to protect from the development of obesity-related inflammation and IR, most likely also due to activity of members of peroxisome proliferator-activated receptor- (PPAR)s (particularly PPAR-α and -γ) and liver X receptor- (LXR) families, molecules involved in nutrient transport and metabolism and able to antagonize inflammatory activity [36, 37]. To contribute to this physiological WAT condition is a new adipokine, the lipocalin-2 (LCN2). LCN2 upregulates PPARγ, increases the release of adiponectin and also antagonizes TNF-α effects on inflammatory and metabolic gene expression in adipocytes and macrophages. Conversely, knocking down LCN2 expression, using lentiviral shRNA gene silencing, results in decreased expression of PPARγ and its target genes, adiponectin, and leptin. Hence, LCN2, seems to act as an antagonist to the effect of inflammatory molecules on inflammation and secretion of adipokines [38].

In obesity conditions, WAT becomes inflamed, state determined by a crosstalk principally between adipocytes and macrophages [8, 16, 21]. The obesity-related inflammatory state occurs in several sequential stages, characterised by a cellular WAT composition remodelling. An increase in number (hyperplasia) and size (hypertrophy) of adipocytes, a macrophage infiltration, and fibrosis characterise WAT in obesity human conditions [39–45]. Adipocyte hypertrophy is induced by two factors: increased fat storage in fully differentiated adipocytes and increased expression of proinflammatory mediators [43–45]. On the other hand, hypertrophic adipocytes shift the immune balance towards the production of proinflammatory molecules [40, 46–49]. The shift in the cytokines profile creates a tissue milieu responsible of the strong modification of the WAT macrophages pool from activated M2 type to classically-activated M1 type [21, 34, 35, 40, 46–49]. In addition, the M1 macrophage WAT pool considerably increases because of the differentiation of monocytes recruited in inflamed WAT. In obese WAT, macrophages also aggregate in "crown-like structures" constituted by necrotic-like adipocytes and adipocyte cellular fragments [46–51]. An increased infiltration of macrophages, indeed, occurs in the inflamed WAT, preferentially into visceral WAT, which contributes of the WAT inflammation state and its exacerbation [10, 16, 17, 20, 21, 34, 35, 46, 47]. Several chemokines, chemokine receptors, and adhesion molecules are involved in this process [50]. It seems also to be directly correlated with both hyperplasia and hypertrophy of adipocytes and inflammatory mediator production [10, 16, 17, 20, 21]. However, the mechanisms responsible for attracting monocyte/macrophage cells and their entry into the fat mass remain unclear. The group of Sengenès et al. has recently evidenced a key role of endothelial cells in the control of the inflammatory WAT process. It has also been described the potential involvement of WAT-endothelial cells as further factors involved in the regulation of macrophage phenotype in the "inflamed fat tissue" [48]. Inflammatory WAT cytokine profile seems to be responsible of the activation of endothelial cells and their

expression of a series of adhesion molecules involved in the recruitment of monocyte/macrophages. Furthermore, it has been demonstrated an association between angiogenesis and adipogenesis [52].

Concerning the shift of cytokines (M2/M1 cytokine profile), the exact mechanisms involved have not yet been clarified [21, 34, 35, 40, 46–49]. It has been proposed that adipocytes from different body depots show differences in their inflammatory phenotype, with visceral fat characterised by more inflammatory phenotype respect to subcutaneous fat, as discussed above [9, 12]. Besides, the increased obesity-associated preadipocyte differentiation process seems also to contribute to this inflammatory cytokine profile [9, 12]. However, human WAT macrophage subsets show no strict M1 or M2 polarization, as recently demonstrated by Bourlier et al. [40] and Zeyda et al. [21].

Furthermore, another characteristic phenomenon, the local hypoxia induced by hypoperfusion due to rapid fat mass expanding seems to contribute to WAT inflammatory state [53–55]. Adipose hypoxia, indeed, induces the release of proinflammatory mediators [53–55].

However, on the whole, these observations give no a clear dissection of the cause and effect relationship between obesity and inflammation. On the other hand, it has, recently, been demonstrated that increased adipose tissue mass is not essentially related with WAT inflammation state, as observed in two studies performed in adiponectin transgenic mice and lipocalin-2 knockout mice [56–58]. This suggests that, although obesity is directly linked to inflammation, the specific role of adipokines and related pathways might be clarified.

3. Molecules Produced by WAT: "Adipokines"

WAT releases hundreds of biologically active molecules, the "adipokines", including more than 50 cytokines, chemokines, hormone-like factors and other mediators [8, 18, 19] Not exclusively produced by WAT cells, these mediators are released by other different body's tissues and organs with functions unrelated to those within WAT [18, 19]. Adipokines affect appetite and satiety, glucose and lipid metabolism, blood pressure regulation, inflammation and immune functions [8, 18, 19]. Precisely, they work as a network to regulate inflammation, insulin action, and glucose metabolism locally and systemically. This adipokine/cytokine networking system is altered in obesity, contributing to inflammation state and impaired adipocyte metabolism. However, how adipokines and cytokines coordinately regulate obesity-related inflammation and metabolism is not clearly understood [8, 18, 19].

Different mechanisms are involved in the adipokine secretion. The production of inflammatory adipokines (such as proinflammatory cytokines, chemokines, molecules associated with thrombosis, and hypertension, etc.) seems to be complex and involves several inflammatory pathways, activated by both extracellular mediators and intracellular stressors. Among extracellular factors, the FFA are the primary inductors of these pathways [59]. In human obesity, they are chronically elevated (by determining lipotoxicity), because of blunted incapacity of insulin to inhibit the lipolysis and the excessive consumption of dietary lipids [60].

Innate immunity receptors, such as Toll-like receptor (TLR)-4 and -2, are expressed in WAT (particularly by adipocytes, preadipocytes, macrophages, and endothelial cells) and are involved in this obesity-related inflammatory process. Their expression is increased and induced in obese subjects [60, 61]. FFA and other molecules produced by hypoxic conditions during obesity activate these receptors, particularly TLR4 [60, 61]. Lipopolysaccharide (LPS) is another factor able to activate TLR4 [62]. A key source of LPS is the gut microbiota [59]. It is continually produced within the gut by death of Gram-negative bacteria and is absorbed into intestinal capillaries to be transported by lipoproteins [63, 64]. On the other hand, it has been observed that a high-fat diet given to mice increases the proportion of gut LPS [63, 64]. These data indicate the gut microbiotia may have an important role in the induction of chronic obesity-related inflammation [63].

Hence, FFA, particularly via TLR4 induce the pro-inflammatory adipokine production in adipocytes [65, 66]. FFA also activate macrophages, referentially of the CD11c+ subset, through the TLR4 pathway, exacerbating their pro-inflammatory activity [66–68]. Furthermore, a paracrine loop between hypertrophied adipocytes and macrophages has been evidenced, able to induce a vicious circle of inflammatory exacerbated WAT state [68]. This paracrine loop involves FFA and TNF-α. As a result, macrophages secrete the pro-inflammatory TNF-α. TNF-α in turn, acting particularly on TNF-α receptor 1 subtype, induces inflammatory changes in hypertrophied adipocytes as well as increased release of FFA [68]. In addition, this paracrine cross-talk could be further improved in obese subjects through the adipocyte hyperresponsiveness to TNF-α and subsequent hyperactivation of inflammatory pathway [52]. FFA may also be active on the adipocyte in an autocrine way to evoke an inflammatory state and chemokine/adipokine overproduction at least in part via TLR4 [69]. This autocrine mechanism has been proposed to be an initial event of the inflammatory WAT cascade, but this issue is still controversial.

In obese WAT the cells and the intracellular organelles are also exposed to increased stress, mainly as a result of metabolic overload [31]. In particular, mitochondria and the endoplasmic reticulum appear to be the most sensitive organelles to metabolic stressors [70]. In addition, the development of hypoxic conditions in the expanded WAT during obesity results in an increased production of reactive oxygen species and the corresponding development of oxidative stress [70].

Signals mediated by both extracellular and intracellular factors culminate predominantly in the activation, principally via TLR4 receptor, of NF-κB transcriptional factor, responsible of the production of inflammatory mediators, as well as the direct inhibition of insulin signaling [60, 61, 71]. Hence, NF-κB pathway represents the crucial and major factor responsible of obesity-induced inflammation. To amplify inflammation-mediated inhibition of insulin action

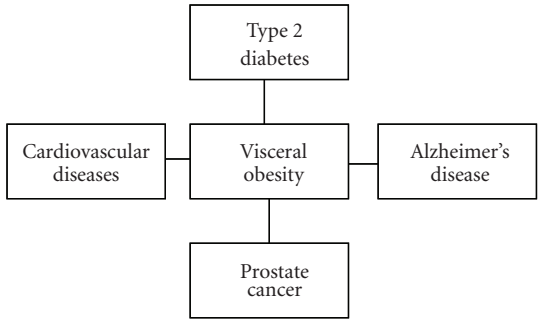

Figure 1: A common yet preventable risk factor for these multifactorial age-related diseases is the visceral obesity. Metabolic syndrome is also associated with obesity. It assembles some abnormalities, including insulin resistance, hyperinsulinemia, hypertension, and dysplipidemia, all risk factors directly associated with both type 2 diabetes and cardiovascular diseases.

are also other pathways, such as those mediated through the suppressor of cytokine signaling-(SOCS)-proteins and iNOS [72, 73].

In contrast, anti-inflammatory adipokine molecules seem to be released through the activation of different transcription factors induced via PPAR-γ and LXR receptors, as mentioned above [36–38]. In conditions of overnutrition, fatty acid-binding proteins, FABPs, likely sequester ligands of PPAR-α and LXRs and induce no activation of these transcription factors [74].

In this paper, the attention has particularly been focussed on adipokines involved in the obesity-related inflammatory diseases. The biological effects of metabolic and inflammatory adipokines are reported in Tables 1 and 2.

4. Role of Visceral Obesity in Ageing and Obesity-Related Inflammatory Diseases

Several pathologies are associated with obesity, such as type 2 diabetes and cardiovascular diseases (CVD) [6]. More recently, the obesity-related risk has also been extended to cancer, including prostate, breast, liver, kidney, colon, ovarian and endometrial cancers [75–81]. The obesity-related diseases are characterised by inflammatory pathophysiology induced by several risk factors (environmental stressors, genetic factors, etc.) and an onset generally correlated with ageing process. Epidemiological studies have revealed that a common yet preventable risk factor for these diseases is the increase of the visceral fat, a characterised hallmark of ageing in humans (Figure 1) [8, 11, 12, 82]. Using either waist circumference and/or waist-to-hip ratio as a proxy of visceral obesity, the role of visceral fat as stronger risk factor for these diseases, than BMI or other fat depots, has been confirmed.

Obesity-related risk is not only limited to these diseases, but also to cognitive decline, Alzheimer's disease (AD) and disability, as recently demonstrated [6, 83–85].

Recent evidence also reports an association of obesity with increased risk of disease specific and all-cause mortality, and with a reduced life expectancy [82]. For example, the group of Fontaine reported that Caucasian men and women with a BMI > 40 and age range 20–29 years, could expect a reduction in remaining years of life expected by approximately 6 and 12 years, respectively [86]. An increment of mortality and a reduction of life expectancy correlated with obesity, especially in old subjects, has been, indeed, proposed. On the other hand, obesity, and precisely visceral obesity, seems to accelerate ageing process. It has been demonstrated that obese women have telomeres of 240 bp shorter lean women of a similar age [87]. In view of the hypothesis that telomere length in vivo represents cellular turnover and exposure to oxidative and inflammatory damage, this difference in telomere length between being lean and being obese might correspond to 8.8 years of ageing [87].

The increased evidence on visceral obesity, as a stronger predictor of these diseases, has led to assess the mortality risk correlated with abdominal obesity [88–90]. In 2008, a large European study reported that both general (BMI) and abdominal adiposity (waist circumference; waist-to-hip ratio) are strong predictors of mortality risk [91]. However, the importance of visceral obesity was most remarkable among persons with a low BMI [91].

The question of visceral fat, as factor capable of reducing life expectancy, has been recently clarified in an animal model study [92]. The extrapolation of its data in humans suggests the key role of visceral fat and the possibility through its depletion to favour the survival and, hence, longevity, as demonstrated in calorie restriction rats [92]. In human beings, its beneficial effects might be greater, since humans visceral fat depots have direct portal access and, so, a greater potential to harm the liver [82, 92]. This has recently led Huffman and Barzilai to hypothesise the presumed link of visceral fat with both age-related diseases and lifespan. Accumulation of visceral fat represents a greater risk for the development of IR and other processes of metabolic syndrome than other fat depots due to its anatomical location, high lipolytic rate and secretion of inflammatory adipokines. This determines some specific perturbations to tissue including hepatic IR, impaired glucose uptake by skeletal muscle and increased basal lipolytic rate and WAT free fatty acid release. The long-term consequences are an increased risk for obesity-related inflammatory diseases and mortality and a reduced lifespan [82].

4.1. Evolutionary Speculations about the Link between Obesity and Obesity-Related Diseases. The association of obesity with obesity-related inflammatory diseases might be explained through evolutionary speculations. It is appropriate, hence, to consider the fundamental biological necessities for the survival of an organism: (1) the ability to resist to starvation and (2) to evoke an efficient immune response to pathogens. To this aim, several metabolic and immune pathways and nutrient- and pathogen-sensing systems have been selected and evolutionarily conserved throughout species. The metabolic systems have been selected to assure energy efficiency through the storage of excess calories particularly under intermittent food uptake conditions. In contrast, under continuous overnutritional conditions, these systems

Table 1: Adipokines involved in energy balance/metabolism.

Name	Cell type expression	Biological effects
Leptin: no glycosylated peptide hormone of 16 kDa encoded by the obese (ob) gene, discovered in 1994 by Zhang et al. [18, 19]	*Adipocytes*: synthesis induced by food intake, eating-related hormones, energy status, and sex hormones (being inhibited by testosterone and increased by ovarian sex steroids) and several proinflammatory mediators (being increased or inhibited by proinflmmatory cytokines with acute or chronic action)	Satiety signal with direct effects on the hypothalamus; stimulates lipolysis; inhibits lipogenesis; improves insulin sensitivity; increases glucose metabolism; and stimulates fatty acid oxidation. Hence, leptin operates as adipostatin and general inductor of energy reserve, being involved in glucose metabolism, synthesis of glucocorticoids. However, it is also known its involvement in other processes, such as the proliferation of lymphocytes (particularly CD4+) and induction of Th1 response, cytokine production, phagocytosis, and regulation of hypothalamic-pituitary-adrenal-axis, reproduction, angiogenesis, and oxidative stress. Collectively, these functions consent to define leptin as a cytokine-like hormone characterised by pleiotropic propriety [18, 19]
Adiponectin: a protein of 30-kDa with a structural homology with collagen VIII and X and complement factor C1q. Once synthesised, it forms trimers which then oligomirize to constitute polymers composed of 4 to 6 trimers. Trimers, hexamers, and high molecular weight (HMW) 12 to-18 mers of adiponectin are present in circulation [18, 19]	*Adipocytes*	Increases fatty acid oxidation with reduction in plasma fatty acid levels; decreases plasma glucose levels; increases insulin sensitivity; anti-inflammatory, antioxidant, antiatherogenic and anticancer properties through the inhibition TNF-α-mediated of NF-κB pathway
Resistin: a member of resistin-like molecule (RELMs) family, called "resistin" for its capacity to induce IR in mice. Resistin is also known as member of molecule "found in inflammatory zone" (FIZZ)—family characterised by four members, characterised by a conserved 11-cysteine pattern at the C terminus. Resistin or FIZZ-3 was initially discovered in mice [18, 19]	*Adipocytes and M2 macrophages*	Induces severe hepatic insulin resistance-increased rate of glucose production in rat (increased resistin plasma concentrations in diet-induced obese mice, but reduced mRNA levels in WAT of obese rodents; stimulates lipolysis); functions controversial in humans
Adipsin: (also called in human complement factor D46) is a rate-limiting enzyme in the alternative pathway of complement activation [18, 19]	*Adipocytes and M2 macrophages*	Stimulates triglyceride storage, inhibits lipolysis
Apelin: a bioactive peptide, representing endogenous ligand of orphan G-protein-coupled receptor, APJ, homolog to angiotensin II receptor [18, 19]	*Adipocytes and stromal vascular cells (in particular macrophages)*	Reduces food intake (?); inhibits glucose-induced insulin secretion; antagonize angiotensin II effects in atherosclerosis inducing NO production and inhibiting angiotensin II cellular signaling (? However, there are contrasting literature data).
Visfatin: An insulin mimetic adipokine recently discovered and released prevalently by visceral WAT. Visfatin is also identical to pre-B-cell colony-enhancing factor (PBEF), a cytokine that has been observed increased both in bronchoalveolar lavage fluid in animal models and in neutrophils in septic conditions Under endotoxin stimulation, PBEF/visfatin is produced by neutrophils, inhibiting neutrophil apoptosis [18, 19]	*Adipocytes Under endotoxin stimulation, PBEF/visfatin/NAMPT is also produced by neutrophils, inhibiting neutrophil apoptosis*	Insulin-mimetic effects; hypoglycaemic effects by stimulating glucose uptake; promotes insulin sensitivity; proadipogenic and lipogenic action. It also induces chemotaxis and the production of IL-1β, TNF-α, and IL6 in CD14+ monocytes and increases proliferative responses in lymphocytes. In addition, visfatin seems to have a nicotinamide adenine dinucleotide (NAD) biosynthetic activity in pancreatic β cells [49]. Hence, visfatin acts as nicotinamide phosphoribosyltransferase (Nampt), the rate-limiting enzyme that converts nicotinamide (a form of vitamin B3) to nicotinamide mononucleotide (NMN), and a NAD precursor [51].

Table 1: Continued.

Name	Cell type expression	Biological effects
Vaspin: a serpin (serine protease inhibitor) [18, 19]	*Adipocytes*	Improves insulin sensitivity; suppresses the production of resistin, leptin, and TNF-α
Omentin: a secretory protein, recently identified as a new adipokine It is codified by two genes (1 and 2) [18, 19]	*Stromal vascular cells (in particular macrophages)*	Enhances insulin-stimulated glucose transport in subcutaneous as well as omental adipocytes; modulation of insulin action
Lipocalin-2 (LCN2), also known as 24p3 or neutrophil gelatinase-associated lipocalin (NGAL): a recently indentified adipokine of the superfamily of lipocalins. It is a 25kDa secretory glycoprotein, originally identified in mouse kidney cells and human neutrophil granules. In adipose tissue, it is highly expressed in vivo and in vitro, and its secretion is regulated by the activation of inflammation and infection [58].	*Adipocytes and macrophages, but also neutrophils, hepatic and kidney cells*	Has different actions, such as apoptosis and innate immunity; affects glucose metabolism and insulin sensitivity; seems to have dual effects on inflammation: pro- and anti-inflammatory effects. So, increased levels of LCN2 in obesity and IR may constitute a protective mechanism against inflammation [58]
Retinol binding protein-4 (RBP4): this protein belongs a the superfamily of lipocalins. And it is a specific carrier for retinol in the blood [58].	*Adipocytes*	Promotes IR and the type 2 diabetes [58]

induce a fat excess, as no advantageous state associated with the onset of several chronic disorders, such as obesity and its complications. At the same time, the necessity to defend against the infections has determined the selection of strong immune components, particularly induced by the epidemic and pandemic infections [93, 94]. The combination of these efficient systems has likely created a fundamental biological instrument able to store energy and to evoke immune-inflammatory responses. Its existence is showed by the several pathways with metabolic and immune functions evolved from common and ancestral units. A characteristic ancestral unit is the body fat of *Drosophila*, having metabolic functions and an intimate control of immune responses. It has given rise after about 600 million years to homologous mammalian tissues, such as haematopoietic and immune systems, liver and adipose tissue [95–98]. These mammalian tissues have conserved their development heritage and show metabolic and immune cellular components with an architectural organization able to have immediate access into blood vessels. Hence, they have continuous and dynamic interactions with other important metabolic and immune sites, such as pancreas. This contributes to understand the mechanisms involved in metabolic diseases, such as type 2 diabetes [99]. They have common regulatory molecular pathways and pathogen-sensing systems able to regulate both metabolic and immune functions. Characteristic example is the TLR4-NF-$\kappa\beta$ pathway, evolved by Drosophila homologous Toll and able to mediate efficient immune responses also metabolically or nutritionally induced and lipid-related pathways able to respond to the energy necessities of particular conditions, such as during the induction of immune or inflammatory response [66, 93, 95].

These observations suggest a fine balance between metabolic and immune systems. Its dysfunction is dangerous and responsible of the development of some diseases. In particular, overnutritional conditions, fruit of current nutritional habits and lifestyles of most modern Western populations, are responsible of the development of the obesity-related inflammatory diseases [100].

In the light of these observations and suggestions, we described the role of adipokines in the pathophysiology of obesity-related inflammatory diseases.

5. Adipokines and Obesity-Related Inflammatory Diseases

5.1. Metabolic Syndrome and Cardiovascular Diseases. The term "Metabolic syndrome" (MS) assembles some abnormalities, including visceral obesity, dyslipidemia, hyperglycaemia and hypertension. The criteria to define MS have been established by International Diabetes Federation (IDF) [97]. In the IDF consensus, MS is defined by the presence of visceral obesity plus two of the described components [101, 102]. The presence of any two of the four next components is also required: elevated circulating levels of triglycerides, reduced levels of HDL-cholesterol, high blood pressure and impaired fasting glycaemia [101, 102]. The IDF eventually recommends other criteria to diagnose MS, such as increased levels of circulating inflammatory and/or thrombotic markers (CRP, SAA, TNF-α, IL-6, and PAI) or reduced levels of anti-inflammatory molecules, such as adiponectin. This syndrome is currently considered one of the major public health challenges, as demonstrated by two large studies performed, respectively, in 2.600 American individuals (age range 25–64 years) and 3000 European subjects (age range ~55 years) [103]. In the two populations, the 25%–40% and 30% (of an Italian cohort), respectively, were affected by MS [103].

The MS pathophysiology is complex and different adipokines seem to be involved. Several reports have demonstrated in MS patients increased IL-6 levels related to BMI

Table 2: Adipocytokines, chemokines, vascular proteins, and other proinflammatory markers produced in WAT and systemic sites and involved in the inflammatory-obesity responses.

Name	Cell type expression	Biological effects
Proinflammatory cytokines		
IL-6: one the crucial pro-inflammatory mediator, secreted by several body's cell types (monocytes, adipocytes, endothelial cells, fibroblasts, etc.) [10, 11]	*Stromal vascular fraction*: the 90% WAT IL-6 production comes from these cells. Under the obesity conditions, both adipocytes and macrophages are the principal responsible of WAT derived IL-6, although the stimuli for the induction of IL-6 production seem to be different.	Decreases insulin and leptin signaling; induces the hepatic release of acute-phase proteins, such as C-reactive protein, and the hypothalamic induction of fever; seems to have a controversial role in insulin resistance: it seems to impair hepatic signaling through the increased expression of SOCS-3 impairing the phosphorylation of insulin receptor substrate 1 (IRS-1) and the transcription factor PKB/Akt. Furthermore, down-regulates the expression of IRS-1 and Glucose transporter 4. In addition, SOCS-3 has the capacity to bind and to inhibit the insulin receptor and to induce the proteosomal degradation of IRS proteins. IL-6 also induces fatty acid oxidation and lipolysis [102]
TNF-α: another remarkable proinflammatory cytokine [10, 11]	*Adipocytes and M1 macrophages*	Induces IR and increases lipolysis in adipocytes; decreases adiponectin and increases IL-6 expression. TNF-α should also play an atherogenic role inducing an increased expression of adhesion molecules in vascular wall, increasing the scavenger receptor class A expression and oxidised LDL uptake in macrophages and stimulating their infiltration in vascular wall
IL-1: Another pro-inflammatory cytokine, member of IL-1 family (IL-1α, IL-1β, and IL-1Ra) [10, 11]	*M1 Macrophages*	Induces fever, acute-phase proteins, proliferation of fibroblasts, smooth muscle cells, and production of antibodies, cytokines, and angiogenesis, metastasis, and cartilage breakdown. It also appears to affect glucose homeostasis and insulin sensitivity through central and peripheral mechanisms. IL-1 also mediates direct effects on adipocytes, decreasing the expression and the activity of LPL, increasing lypolisis and affecting adipocyte differentiation through inhibition of PPAR receptors
Anti-inflammatory cytokines		
IL-1Ra: a cytokine antagonist able to limit inflammation, competing with IL-1 for binding to its receptor without inducing a signal [10, 11]	*M2 macrophages and hepatic cells as an acute-phase protein under systemic inflammation stimuli*	Produced in response to stress and by M2 macrophages to create an anti-inflammatory WAT milieu in physiological condition. High serum levels of IL-1Ra are associated with insulin resistance
IL-10: an anti-inflammatory cytokine inhibiting the production of several proinflammatory cytokines (IL-1, IL-6, and TNF-α), chemokines and increasing the levels of anti-inflammatory cytokine such as IL-1Ra [10, 11]	*Adipocytes and M2 macrophages*	Produced by M2 macrophages to create an anti-inflammatory WAT milieu in physiological condition. In obesity, high levels of IL-10 have been observed
Proinflammatory chemokines		
IL-8: a proinflammatory chemokine [10, 11]	*Stromal vascular cells*	Induces the migration of different cell blood types, such as monocytes, particularly in inflammatory conditions. In obesity, high IL-8 levels have been observed and increased levels of IL-8 mRNA have been detected principally in visceral WAT. They seem correlated to fat mass and BMI
Mcp-1 (CCL2): key chemokine involved in recruitment of monocytes/macrophages and in monocyte tissue infiltration. Its levels conspicuously increase under IL-1, TNF-α, and LPS stimuli, while under normal conditions are undetectable [10, 11]	*Adipocytes/M1 macrophages*	Increases lipolysis and leptin secretion; decreasesinsulin-stimulated glucose uptake; (increased plasmaconcentrations in obesity; disturbs insulin sensitivity)

Table 2: Continued.

Name	Cell type expression	Biological effects
Adipokines associated with thrombosis and hypertension and other inflammatory markers		
PAI-1: a serine protease inhibitor (serpin) with the physiological function to inhibit plasminogen activation [10, 11]	*Stromal vascular cells with visceral WAT secretion more elevated than subcutaneous WAT*	Inhibits plasminogen activation. Elevated PAI-1 levels determine a pathological condition characterised by hypofibrinolysis and a prothrombotic state It affects cellular matrix degradation, smooth muscle cell migration and angiogenesis, determining the development of atherosclerosis. In obese conditions, PAI-1 seems to contribute directly to obesity complications, such as atherothrombosis, insulin resistance and type 2 diabetes
Angiotensinogen (AGT): the precursor of vasoactive peptide angiotensin II (Ang II), a component of vasoconstrictor renin-angiotensin system (RAS) [10, 11]	*Stromal vascular cells and adipocytes, with visceral WAT secretion more elevated than subcutaneous WA*	Linked to vascular inflammation (increased plasmalevels in obesity) and increased blood pressure
C-reactive protein (CRP): one of the acute-phase proteins in inflammation. It is a member of short pentraxins produced in the liver in response of IL-6 [10, 11]	*Hepatic cells, human mature adipocytes, but not preadipocytes, under inflammatory stimuli, including lipopolysaccharide (LPS), TNF-α, and resistin*	Endothelial dysfunction, adhesion molecules expression, Tissue factor production, PAI-1upregulation, mononuclear cells recruitment, adhesion, activation and cytokine production, ROS and MMPs production, uptake of oxLDL, foam cells formation, proliferation, migration, ROS production, MMPs, MCP-1, and iNOS expression
Serum amyloid protein (SAA): constitute a family of lipoproteins involved in the transport of cholesterol and the host defence alarm system [10, 11].	*Hepatic cells, adipocytes*	SAA are not only inflammatory markers induced by IL-6, but also represent inflammatory mediators able to induce inflammatory events in leucocytes. In particular, SAA proteins can mediate chemotaxis of monocytes into WAT with hypertrophic adipocytes and at the same time to increase the expression of adhesion molecules in endothelial WAT cells. SSA proteins seem responsible of increased incidence of cardiovascular diseases in obese individuals. They are able to interact with high-density lipoprotein (HDL)-receptor competing with HDL, and thereby inhibit the HDL-mediated clearance of cholesterol, increasing the development of atherosclerotic lesions.

[100, 101]. In particular, the involvement of IL-6 in IR and its complications has been evidenced, even if its role remains controversial [104–106]. The mechanisms involved, indeed, are not fully clear. However, IL-6 seems to induce IR, impairing hepatic signaling through the increased expression of SOCS-3 and affecting the phosphorylation of insulin receptor substrate 1 (IRS-1) and the transcription factor PKB/Akt [104–106]. SOCS-3 has the capacity to bind and to inhibit the insulin receptor and to induce the proteosomal degradation of IRS proteins. Using 3T3-L1 adipocytes, it has been demonstrated the IL-6 capacity to induce partial resistance in insulin-dependent glucose uptake through down-regulation of the phosphorylation of IRS-1 and the expression of IRS-1 and Glucose transporter 4 (GLUT–4) [10, 66–68, 107]. Furthermore, in 3T3-L1 adipocytes, IL-6 reduces the activity of lipoprotein lipase-LPL [52, 107].

TNF-α seems also involved in MS. High TNF-α levels have been observed in MS subjects [100]. The relationship between high levels of TNF-α and MS is related to the TNF-α capacity to induce a c-Jun NH2-terminal kinase to mediate a serine phosphorylation of IRS-1. This determines the inhibition of normal tyrosine phosphorylation of IRS-1 and downstream insulin signaling [104].

Furthermore, in obesity, an overexpression of angiotensinogen and an increased activity of vasoconstrictor renin-angiotensin system have been demonstrated [108]. This phenomenon seems also to contribute to the alteration of insulin sensitivity and to increase the incidence of type 2 diabetes and MS [109].

Recent evidence also demonstrates the association of elevated levels of systemic inflammatory molecules, such as SAA and CRP, with type 2 diabetes and MS [110].

It has also been demonstrated the role of leptin in the MS pathophysiology. It evokes a condition, which affects insulin sensitivity and induces IR. In particular, leptin induces in hypothalamus the release of "anorexigenic peptides" (i.e., proopiomelanocortin and corticotrophin-releasing hormone) and, reciprocally the inhibition of

"orexigenic peptides" (i.e., neuropeptide Y and agouti-related protein), thereby limiting food intake [111]. In obesity conditions, hypothalamic resistance to leptin has been found and ascribed to reduced transport of leptin across the blood-brain barrier and to increased levels of SOCS-3 and ER stress, which inhibit leptin signaling [112, 113]. This evokes profound changes in energy balance and hormone production via the hypothalamus, analogous to those induced in response to fasting. Hence, a response of adaptation to low levels of leptin is induced, determining overfeeding and inhibition of energy expenditure, thyroid and reproductive hormones, and immunity. Hypothetically, this response may be evolved as a protection against the threat of starvation [111]. In obese subjects, these changes determine reduced energy expenditure and to regain weight, associated with lipid accumulation [114]. Ectopic lipid storage (in liver, epicardial, and muscle fat) is also induced in obese conditions because of leptin resistance, which may in turn further impair insulin sensitivity [52]. Like leptin effects, another adipokine, resistin, seems to mediate IR. Its role may be evidenced in rodents, as suggested by an interesting theory [18, 19, 52]. However, successive studies in both rodents and humans have reported contradictory data [18, 19].

An interesting role in the MS pathophysiology and its complications seems mediated through a more recent discovery adipokine, visfatin, which its plasma levels are correlated to lipid metabolism and inflammatory response [18, 115]. It mediates a nicotinamide adenine dinucleotide (NAD) biosynthetic activity in pancreatic β cells [116]. Hence, visfatin acts as nicotinamide phosphoribosyltransferase (Nampt), the rate-limiting enzyme that converts nicotinamide (a form of vitamin B3) to nicotinamide mononucleotide (NMN), a NAD precursor [112]. It has been reported a decline with advanced age of Nampt-mediated systemic NAD biosynthesis, able to determine a reduced sirtuin-1 activity. This mechanism might contribute to decreased function of pancreatic β cells in aged subjects [117].

Furthermore, another adipokine recently identified with a key role in insulin resistance is LCN-2, as demonstrated in LCN-2 knockout mice [57].

In contrast, a protective role of adiponectin against MS (and the other obesity-related pathologies) has recently been demonstrated [118]. This molecule reduces M1 macrophage functions, by inhibiting phagocyte activity and release of IL-6 and TNF-α, and increases the IL-10 and IL-1Ra production in adipocytes and macrophages [10, 18, 19]. Apelin also reduces the MS risk. In obesity, increased plasma and WAT levels of apelin have been detected [18, 19]. TNF-α seems to be the responsible of these increased levels both in plasma and WAT [18, 19]. This molecule seems to reestablish glucose tolerance and increased glucose utilization, as demonstrated in a mice study [119]. These data should suggest the use of this molecule in the treatment of IR. Studies on animal models particularly in the apelin-knockout mice have evidenced that loss of apelin determines heart diseases in response to pressure overload [120].

Growing evidence highlights the link of systemic-obesity inflammatory state with both CVD onset and CVD risk [110, 121]. Several studies have demonstrated a link between WAT excess and CVD mortality in young (particularly in adolescents) and old subjects [110, 121]. Furthermore, a relationship between WAT excess and coronary artery calcium (a marker of coronary atherosclerosis) measurement has been reported [122]. Imaging approaches have also been confirmed this association [122]. How obesity determines the CVD development, it is until now not clearly understood. Complex and numerous obesity-mediated mechanisms are identified, as well as the CVD risk obesity-related factors (including hypertension, IR and dyslipidemia). Systemic and WAT adipokines seem also to affect vessel wall, by determining adverse effects [110, 121, 122]. Precisely, proinflammatory cytokines, hormone-like molecules and other WAT adipokines act in the liver, causing changes in the production and the release of lipoproteins, coagulation factors and inflammatory molecules [110, 121, 122]. In particular, they induce an increase of very-low-density lipoprotein, apolipoprotein B (apoB), and triglyceride secretion [123]. These liver-released molecules act on endothelial, arterial smooth muscle and macrophages cells, by inducing atherogenic effects on the vessel wall through the regulation of their gene expression and functions [110, 121, 122]. In addition, visceral fat seems to be particularly involved in the activation of these pathways [122, 123].

Interestingly, among adipokines, leptin mediates different effects on cells of vessel wall. It evokes on endothelial cells oxidative stress, increased production of adhesion molecules and chemokines and proliferation [110, 121, 122]. For example, an increased blood release of MCP-1 in obese condition has been observed. It seems to increase the number of CD11c+ monocytes, favouring the binding of monocytes/macrophages to the vessel wall [124]. Acting also on smooth muscle cells, leptin induces their migration, proliferation, and hypertrophy [110, 121, 122]. It also induces a further activation and cytokine production of macrophages, neutrophils, and T cells and it seems also involved in the calcification of cells of vessel wall and the thrombosis through the increase of platelet aggregation [110, 121, 122]. These effects are also indirectly mediate of leptin through leptin resistance (mentioned above).

Resistin induces similar effects to those of leptin. In human, levels of resistin seem to be positively associated with coronary atherosclerosis [125]. It induces on endothelial cells an increased expression of adhesion molecules, proinflammatory cytokines, and pentraxin [125].On smooth muscle cells, it evokes their migration. An increased expression of CD36 on macrophages seems to be also mediated by resistin, facilitating lipid accumulation and formation of foam cells [126, 127]. On macrophages it also mediates an increased production of proinflammatory cytokines, through via TLR4 and NF-κB pathway [128].

Among the adipokines of recent discovery, visfatin, and apelin seem to have a key role in the CVD pathophysiology. Visfatin has a key role in plaque destabilization, associated with its increased expression in macrophages of human

unstable carotid and coronary atherosclerosis [129]. In contrast, its plasma levels are negatively associated with vascular endothelial function [130]. Paradoxical data reported by van der Veer et al. have demonstrated that visfatin can, however, prolong the life of human smooth muscle cells [131].

Unlike visfatin, apelin is associated with a positive hemodynamic profile and has positive inotropic effects in normal and failing rat hearts and in isolated cardiomyocytes [132–134]. In patients affected by single atrial fibrillation and chronic heart failure, reduced plasma apelin levels have been found [135, 136]. In vessel wall and cardiovascular tissue of rats, apelin production seems to be upregulated by hypoxia and ischemic cardiomyopathy, likely as a compensatory mechanism [137, 138].

In contrast, adiponectin seems to induce beneficial effects. Its levels are positively correlated with HDL levels, and negatively with triglyceride levels, IR, and systemic circulating inflammatory markers [139, 140]. Furthermore, a negative correlation between adiponectin and coronary artery calcium has been observed [141]. It also promotes several anti-atherogenic and anti-inflammatory effects on vessel cells: it downregulates the expression of adhesion molecules on endothelial cells [142]; it decreases endothelial oxidative stress and increases eNOS activity [143]; in smooth muscle cells it inhibits proliferation by suppressing the release of growth factors [144]; and in macrophages it reduces lipid accumulation and the expression of scavenger receptors [145].

Furthermore, CRP, usually increased in CDV, has atherogenic effects on vessel wall [146, 147]. This atherogenic effect is also increased by other WAT molecules, such as AGT, angiotensin-converting enzyme and PAI-1. AGT II has vasoconstrictive actions and also promotes systemic inflammation [121, 122]; AGT contributes to the activation of renin-angiotensin system (RAS) and both these molecules induce a hypertensive response [117, 118]. PAI-1 seems to be involved in atherothrombosis [121, 122].

5.2. Obesity and Alzheimer Disease. AD is a heterogeneous and progressive neurodegenerative disease which in Western societies mainly accounts for clinical dementia [148]. The AD prevalence is below 1% in individuals aged 60 years, but shows an almost exponential increase with age, so that, in the Western world, in people aged 85 years or older the prevalence is between 24% and 33%. It prevalence is expected to quadruple by the year 2047 in the United Stated [149].

There is currently no cure for AD and its pathogenesis remains the subject of many theories involving genetic as well as environmental factors. Recent mounting evidence has been supposed the involvement of modifiable risk factors in AD neurodegeneration, such as lifestyle factors. Among these, obesity represents an AD risk factor. Several potential mechanisms seem to link obesity with AD: hyperglycemia, advanced glycosylation products, adipokine action, and the influence of obesity on vascular risk and cerebrovascular disease.

IR and hyperinsulinemia seem to represent the key causes for the development of some age-related diseases, such as AD [6]. Recently, a role of insulin in AD neurodegeneration has been reported [150]. Precisely, insulin, crossing the blood brain barrier from periphery to central nervous system, seems to compete with Aβ amyloid peptide for insulin degrading enzyme (IDE) in the brain, including also the hippocampus [151]. In contrast, insulin produced in the brain seems to have an advantageous effect on amyloid clearance [152]. Opposing effects seem to be mediated by conditions of peripheral hyperinsulinemia. They seem to determine the inhibition of brain insulin production, which in turn results in impaired amyloid clearance and a higher AD risk [152]. These data suggest that reducing peripheral hyperinsulinemia and increasing brain insulin levels, beneficial effects might be attained on AD neurodegeneration. Therapy strategies able to reduce blood insulin levels in humans have been demonstrated to affect cognition and levels of amyloid β in the cerebrospinal fluid, supporting the potential direct role of insulin in AD [153, 154].

Hyperglycemia seems to be also responsible of the increased levels of advanced glycosylation end products (AGEs). An increased glycation of amyloid β has been demonstrated to improve its aggregation *in vitro*. Furthermore, AGE receptors seem also to be specific cell surface receptors for amyloid β, thus potentially facilitating neuronal damage [155].

Concerning the role of adipokines in AD, it is not clear whether their involvement in the AD pathophysiology are direct or associated with IR and hyperinsulinemia. On the other hand, systemic inflammation seems to be a risk AD factor [156]. Some studies have proposed a direct action of adipokines in AD neurodegeneration. For example, leptin seems to affect CA1 nucleus of the hippocampus [157]. Several effects of leptin on the brain development and potentially on brain health in cognition and ageing have also been observed. This evidences the capacity of leptin to affect the function of the hypothalamus and learning and memory processes controlled by the hippocampus [157]. Leptin receptors have been observed in the hippocampus, hypothalamus, amygdala, cerebellum, and brain stem, supporting its capacity to mediate regulatory mechanisms [157]. A direct interaction between leptin and adiponectin and hypothalamic nuclei has been evidenced [157]. However, other roles of leptin and related adipose-derived factors in the AD brain are not clear [158]. Fasting plasma leptin has been inversely correlated with grey matter volume in areas of the brain in which obese have reduced grey matter in comparison with lean individuals [159].

Another potential link between obesity and AD is cerebrovascular disease (CD). CD and stroke are associated with a higher AD risk [160–162]. Their direct action on amyloid cascade, however, is not clear. Current opinion proposes CD as additional brain damage to amyloid neurotoxicity [161, 162]. However, RAS system seems to link obesity with CD and AD. RAS system regulates the blood pressure. Both human brain and WAT express RAS, with WAT RAS involved in adipocyte growth, differentiation and metabolism [163, 164]. The RAS activation takes place when blood pressure is low. In this state, the formation of Angiotensin II is evoked. Its interaction with specific receptors induces the activation

of RAS, determining an increased of blood pressure. In the brain, angiotensin II continues its conversion to angiotensin IV, which enhances learning and memory in animal models [165, 166].

Another potential mechanism theoretically involved in AD neurodegeneration is hypercholesterolemia. To this aim, some prospective studies have examined total LDL and HDL cholesterol levels as possible risk AD factors. Contrasting data have been reported. The association of cholesterol with dementia may vary depending upon when cholesterol is measured in the life-span and/or relative to the course of disease. High cholesterol may be a risk factor if measured in midlife many years before clinical onset, but then as the disease pathology progresses, cholesterol levels may fall such that it appears that high cholesterol is protective. Two Finnish studies have, indeed, observed that high total cholesterol levels in mid-life are associated with an increased risk of AD more than 20 years later [167, 168]. In contrast, no association has been found cross-sectionally [164]. Two studies each with more than 25 years of follow-up, did not find an association between mid-life total and HDL cholesterol and incident AD [169, 170]. Three other studies in elderly populations also did not find associations between LDL and/or HDL cholesterol and incident AD after follow-ups of 2 years and 7 years [171, 172]. In fact, one of these studies reported an inverse association between total cholesterol and AD, such that those in the lowest quartile had the greatest risk [168]. Similarly, higher cholesterol levels have been reported to be associated with a reduced risk for AD [173, 174].

5.3. Obesity and Prostate Cancer. Prostate cancer (PC) is the most common cancer in Western elderly male populations. Its incidence increases rapidly in men over 50 years of age [175]. The development of PC is based on the interaction between genetic factors and the host exposure to environmental factors, such as infectious agents, dietary carcinogens and hormonal imbalances. In this complex situation, chronic inflammation seems to play a key role [176–181].

As reported above, the risk associated with obesity has also been extended to several malignancies. Its role in PC aetiology is less clear [182]. Data on the association between obesity and PC incidence are inconsistent, and in some studies obesity is associated with an increase in risk of low-grade tumours. The reasons of these contrasting results may be due, in part, to variation of the methods of anthropometric measurement, such as BMI and the waist-to-hip ratio. However, a recent study has revealed visceral fat accumulation as specific risk factor for PC [6, 76]. More consistently, it has recently been suggested that obesity reduces the risk of nonaggressive PC disease and increases the risk of aggressive PC disease [182]. Hence, it is possible that rather than increasing the absolute risk of PC development, obesity may be associated with the progression of latent or microscopic PC to clinically significant and metastatic PC. Furthermore, the differential effects of obesity on PC subtypes suggest aetiological heterogeneity of these tumours and complex interaction between androgen metabolism and several putative risk factors, including IR, diabetes, inflammation, and genetic susceptibility, on PC risk [182].

The molecular mechanisms liking obesity and PC pathophysiology are numerous and occur at several levels. A first mechanism seems correlated to sex steroid pathways [182]. However, the relationship between sex steroid hormones and obesity is complex and biological processes involved are unclear. Current opinion suggests a decline in men of serum testosterone levels in obese conditions [183]. In addition, increased peripheral aromatization of androgens to oestrogens, correlated with fat overload, is also involved in the decline of androgens [182]. On the other hand, it is well-documented an age-related decrease of serum testosterone levels [183]. However, testosterone seems to induce differential effects. Recent data have shown that higher serum levels of total testosterone are associated with a reduced risk of high-grade PC, but with an increased risk of low-grade PC [184]. This emphasizes the complex relationship between obesity and serum sex steroid, and their differential effect on PC, but further supports the differential effect of obesity on PC subtypes.

Another mechanism is correlated to capacity of obesity to modify the production of other hormones, such as insulin-like growth factors (IGFs), having mitogenic properties. An additional mechanism is mediated by adipokines. It has been reported that adipokines may modulate the biological behaviour of PC cells. In particular, leptin, IL-6 and TNF-α seem able to enhance tumour growth [185]. The association between systemic leptin levels and PC has been analysed in several studies. The obtained data have reported a positive association between high leptin levels and the risk of large volume prostatic tumours [185]. Stattin et al. have evidenced an association between moderately high leptin levels and later PC development [185]. Furthermore, the association between leptin and PC seems particularly confined to male subjects having a with waist-to-hip ratio >0.87. This datum evidences the interaction of leptin with other molecules correlated with abdominal obesity, such as sex hormones bioavailability and IGF-1 levels [185]. Another study has demonstrated in a relatively small number of PC patients an association between serum leptin levels and prostate specific antigen and Gleason score [185]. In vitro studies have evidenced a role of leptin in PC carcinogenesis and its capacity to promote the proliferation of androgen-independent PC cell lines [185]. In vitro, it has been also observed the capacity of leptin to induce vascular endothelial cell proliferation, and in vivo angiogenesis, key processes involved in cancer progression, invasion, and metastasis [185]. The proliferative response of PC cells to leptin has been shown to involve intracellular signaling molecules such as phosphatidyl-inositol 3-kinase (PI3-K) and c-Jun NH2-terminal kinase (JNK) [185]. Alterations in these signaling pathways are not only critical in processes of prostate carcinogenesis and malignant transformation, but also important in obesity, diabetes, and IR [185].

High serum IL-6 levels are also associated with PC [185]. A role of IL-6 has been suggested in the early stages of prostate carcinogenesis [185].

Difference effects seem to be mediated by the other adipokines, such as adiponectin proposed as an anticancer factor in some tumours, PC included [181]. In support of this, significant lower levels of adiponectin have been observed in PC patients respect to subjects with benign prostatic hyperplasia or healthy controls [185]. This study also evidences a negative association between plasma adiponectin and Gleason score and PC stage. Adiponectin receptors have been found in both benign and malignant human prostate tissue [185]. In the LNCaP and PC3 PC cell lines, it has been evidenced that androgens, oestrogen, TNF-α, leptin and adiponectin seem all to act and regulate adiponectin receptors. These results might to suggest a complex role of adiponectin in the PC carcinogenesis, mediated through its interaction with sex hormones and cytokines.

6. Conclusions: Possible Strategies for New Therapeutic Treatments for Obesity-Related Inflammatory Diseases

Human visceral obesity represents one of the major risk factors for obesity-related diseases. Possible strategies for the prevention and the development of new therapeutic treatments are, hence, crucial medical challenge.

In researching potential strategies, crucial questions remains open. It is not clear whether effectively obesity inflammatory state determines a metabolic deterioration. Furthermore, it is not also clear whether inflammation can simply be considered a state activated by altered nutrient clearance.

To date, the literature evidence leads to consider the regulator molecular pathways, evolutionary well-conserved and able to control the evocation of immune responses and metabolic processes and, as possible ways for therapeutic approaches. However, their selection is difficult, as well as their manipulation with possible pharmacological agents to interfere with immune and metabolic systems, without to determine severe consequences on key mechanisms of organism. A possible candidate might be the TLR4-NF-κB pathway, having the role of hub in the induction of both metabolic and inflammatory processes, as described. Hence, its antagonists might be used to block the release of metabolic and inflammatory adipokines. On the other hand, TLR4-NF-κB pathway has a key role in the pathophysiology of age-related inflammatory diseases, such as CVD, AD, and PC, as we have recently demonstrated [62, 176, 186, 187].

Other possible pathways might be the lipid-related pathways, such as PPAR-α and LXR pathways. It is already established with success that the use of thiazolidinediones or statins (ligands of this pathway and insulin sensitising compounds) is able to regulate lipid metabolism and to induce anti-inflammatory effects.

Another possible way for the development of possible pharmacological approaches for inflammatory obesity-related diseases might involve the adipokines, even if several their effects and functions remain unclear. In this case, the strategy might hypothetically have as aim the control of the bioavailability of some adipokines, such as leptin and adiponectin, in obese conditions. Exogenous administration of adiponectin might counteract the consequences of obesity state, such as leptin-induced inflammation, or activate its antiatherogenic, vasoprotective and anticancer actions. Another alternative avenue might be the inhibition of leptin receptors through monoclonal antibodies or mutant leptin. Other possible targets might be pro-inflammatory cytokines and chemokines or their receptors, through the use of their agonists or monoclonal antibodies.

In summary, these observations emphasize the necessity to discover the metabolic and immune pathways, including also the molecules involved in metabolic and pathogen sensing systems, involved in the delicate balance of interplay between metabolic and immune systems. This might be useful to clarify and to understand the mechanisms induced and to open possible ways for therapeutic approaches able to enhance the capacity of endogenous molecules to prevent stress and inflammatory responses induced by metabolic signals.

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Research Article

Effect of Moderate-Intensity Exercise on Plasma C-Reactive Protein and Aortic Endothelial Function in Type 2 Diabetic Mice

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The aim of this study was to evaluate the effects of moderate-intensity exercise on plasma levels of C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF-α) as markers of low-grade inflammation and endothelial function in diabetic (db/db) mice. Control and db/db mice were divided into sedentary and exercised groups. Aortic endothelial function was evaluated after two- and six-week exercises using a wire myograph. Plasma CRP levels were measured at baseline, and after two and six weeks of exercise. Baseline plasma CRP levels were significantly higher in db/db mice compared to control ($P < .05$). After two weeks of exercise, aortic endothelial function was significantly improved without affecting body weight or plasma CRP levels. Six weeks of exercise not only improved endothelial function, but also significantly reduced body weight and plasma CRP levels in db/db mice. Thus short-term exercise has beneficial effect on endothelial function without reducing low-grade inflammation while more prolonged exercise periods are required to reduce inflammatory markers.

1. Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in diabetic patients [1], and it is likely that vascular abnormalities may be responsible for the higher incidence of cardiovascular diseases in diabetes. Although it is suggested that endothelial dysfunction is an important contributor to the vascular complications of diabetes [2, 3], the exact mechanisms of impaired endothelial function are unclear.

Lifestyle modification, especially exercise, is routinely recommended for the management of human type 2 diabetes [4, 5]. Exercise is thought to improve vascular function by reducing plasma lipids and blood glucose level [6], oxidative stress [7], and increasing insulin sensitivity [8]. Endothelial dysfunction is one of the earliest events in the progression of cardiovascular diseases.

Chronic low-grade inflammation, as reflected by elevated plasma levels of CRP, is an independent predictor of cardiovascular disease [9, 10] and diabetes [11]. CRP has a number of roles in several cardiovascular diseases [12], and levels of CRP are positively correlated with obesity and insulin resistance [13]. Many studies suggest that a chronic inflammatory process promotes the progression of endothelial dysfunction [14]. In this study, we hypothesized that moderate-intensity exercise improves endothelial function by decreasing low-grade inflammation in db/db mice, a frequently used animal model of type 2 diabetes.

2. Materials and Methods

2.1. Animal Groups. Six-week-old control wild type and diabetic db/db mice (BKS.cg-m +/+ Lepr db/J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All experiments were performed according to the guidelines of the University of British Columbia Animal Care Committee. After one week of acclimatization, animals were randomly divided into four groups ($n = 10$ each): two groups each of control (control sedentary and control exercised) and diabetic mice (diabetic sedentary, diabetic exercised). The

animals were housed ten per cage under conditions of a 12-hour light/dark cycle, 22°C temperature, and with free access to food and water. Body weights were recorded weekly.

2.2. Exercise Program. Mice were exercised using a running wheel (Lafayette Instruments, Lafayette, IN, USA) as previously described in [2, 15]. Mice assigned to the exercise groups were placed in individual running wheels for one hour of daily exercise at a speed of 5.2 m/min (which represents a daily forced exercise of 312 m) for 6 weeks. During the training period (two weeks), mice were exercised daily at a set time each day for 5 days a week. The sedentary db/db or control groups were placed in nonrotating wheels for one hour per day.

2.3. Plasma Variables. Animals were anaesthetized with pentobarbital (50 mg/kg, *i.p.*) combined with heparin (50 U/kg). Blood samples were taken at baseline (6 weeks old), after two weeks of exercise following a two-week training period (the 10th week) and at the end of study (the 14th week). Fasting blood glucose was measured using commercially available kits. Plasma CRP and TNF-α levels were measured using ELISA kits (Alpco Diagnostic, USA).

2.4. Evaluation of Endothelial Function. Thoracic aortas were removed and placed in ice-cold physiological salt solution (PSS) and cleaned of connective tissue. Segments of aorta were threaded with stainless steel wire (0.04 mm diameter) and attached to the tissue holders of a four-channel wire myograph (JP Trading, Aarhus, Denmark). Tissues were allowed to equilibrate for 60 minutes at 37°C during which time the PSS was replaced at 20-minute intervals. During the equilibration period, the resting tension was gradually increased to 5.5 mN and kept at this level for 20 to 30 minutes. Each tissue was maximally activated with a solution of KCl (80 mmol/L) that was prepared by equimolar substitution of NaCl. Following washout with fresh PSS and return of tension to basal preload, phenylephrine (1 μmol/L) was added to establish a stable contraction. Thereafter, cumulative additions of acetylcholine (ACh) (1 nmol/L to 10 μmol/L) were made. Vasodilatory responses were recorded on a computer using MyoDaq Acquisition software (version 2.01; Danish MyoTechnology, Aarhus, Denmark) and expressed as percent dilation of phenylephrine-induced constriction.

2.5. Citrate Synthase Assay. To document the efficacy of an endurance-trained state, citrate synthase activity levels were measured in skeletal muscle. Thigh adductor muscles were gently removed after sacrificing the animal, and citrate synthase activity was measured as previously described in [16].

2.6. Drugs and Chemicals. Acetylcholine, and phenylephrine were purchased from Sigma Chemical Co (St. Louis, MO). The composition of the PSS (mM) was NaCl (119), KCl (4.7), KH_2PO_4 (1.18), $MgSO_4$ (1.17), $NaHCO_3$ (24.9), EDTA (0.023), $CaCl_2$ (1.6), and dextrose (11.1). Isotonic substitutions (replacement of Na^+ with equimolar concentrations of K^+) were used when using PSS solutions with increased K^+ concentrations.

2.7. Statistical Analysis. Results are expressed as mean ± SEM. Data analysis was done using NCSS-2000 software and GraphPad Prism (version 3.02-2000). ANOVA with multiple comparisons was performed using the Bonferroni's test. Correlation analysis using Spearman coefficient tests were performed where appropriate. $P < .05$ was considered as being statistically significant.

3. Results

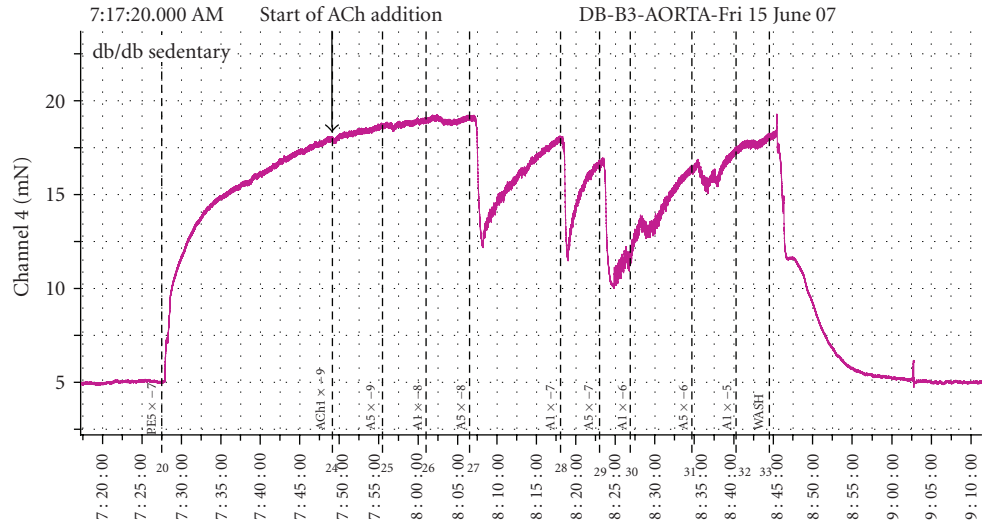
3.1. Body Weight, Blood Parameters, and Effect of Exercise. Six-week old diabetic mice had higher body weights than control mice. After six weeks of exercise, db/db exercised had lower body weights compared to the sedentary group (Table 1). Analysis of baseline blood parameters (6 weeks old), after two weeks (10 weeks old) and six weeks (14 weeks old) of exercise are shown in Table 1. Diabetic mice had higher fasting blood glucose levels at all time points, and while two weeks exercise did not alter blood glucose levels in db/db mice, six weeks of exercise reduced blood glucose levels in diabetic mice ($P < .05$).

Baseline plasma CRP levels were higher in db/db mice compared to control (3.81 ± 0.23 versus 1.83 ± 0.30) ($P < .05$). Plasma CRP levels in db/db mice were not affected by two weeks of exercise but were significantly reduced after 6 weeks exercise (at the 14th week) (3.59 ± 0.41 versus 5.12 ± 0.25) ($P < .05$). Plasma levels of CRP were significantly correlated with body weight ($r = 0.5855$, $P < .0001$) and blood glucose ($r = 0.4821$, $P = 0.0003$) when analyzed by the Pearson test.

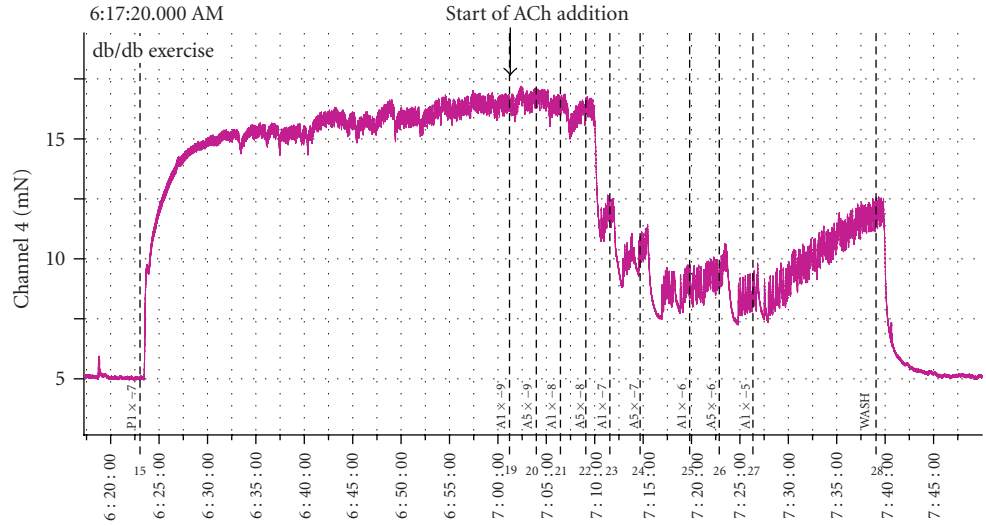
The level of plasma TNF-α in sedentary db/db mice at 14 weeks old (18.62 ± 2.11 pg/mL) tends to be higher than in control mice (14.88 ± 0.35 pg/mL); however, it does not reach statistical significance ($P > .05$).

3.2. Endothelial Function. Acetylcholine (ACh) was used to evaluate endothelial-dependent vasodilatation. Responses to ACh vasodilation were impaired in aortic rings from db/db mice compared with control counterparts (Figure 1). Moderate-intensity exercise in db/db mice for either two or six weeks restored endothelium-dependent vasodilation (Figure 1). The maximal vasodilatation (% loss of induced tone) and sensitivity (EC_{50}) is shown in Figure 2.

3.3. Citrate Synthase Activity. As shown in Table 2, tissue levels of citrate synthase activity were significantly increased in the thigh adductor muscles of db/db and control exercised mice compared to the sedentary groups at both time points (after two and six weeks of exercise) ($P < .01$, $n = 10$).



(a)



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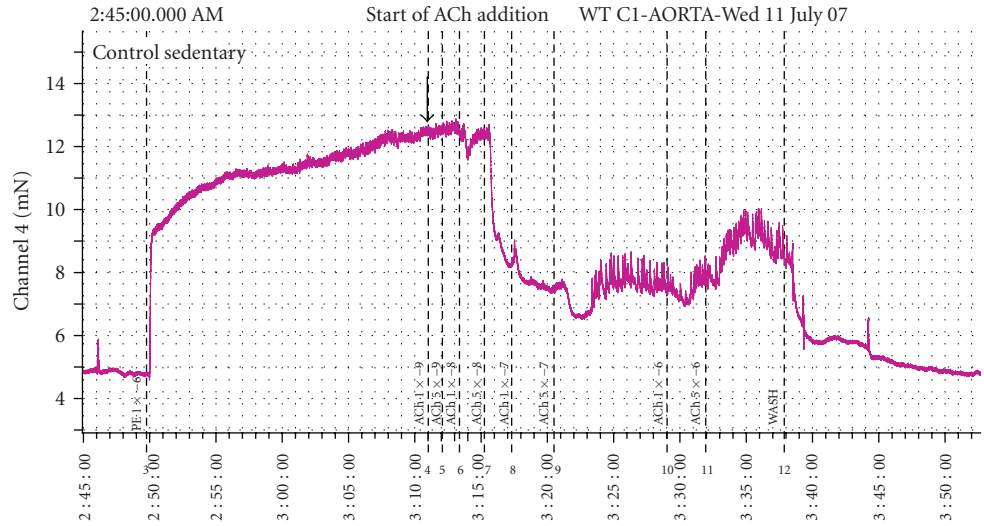

(c)

Figure 1: Continued.

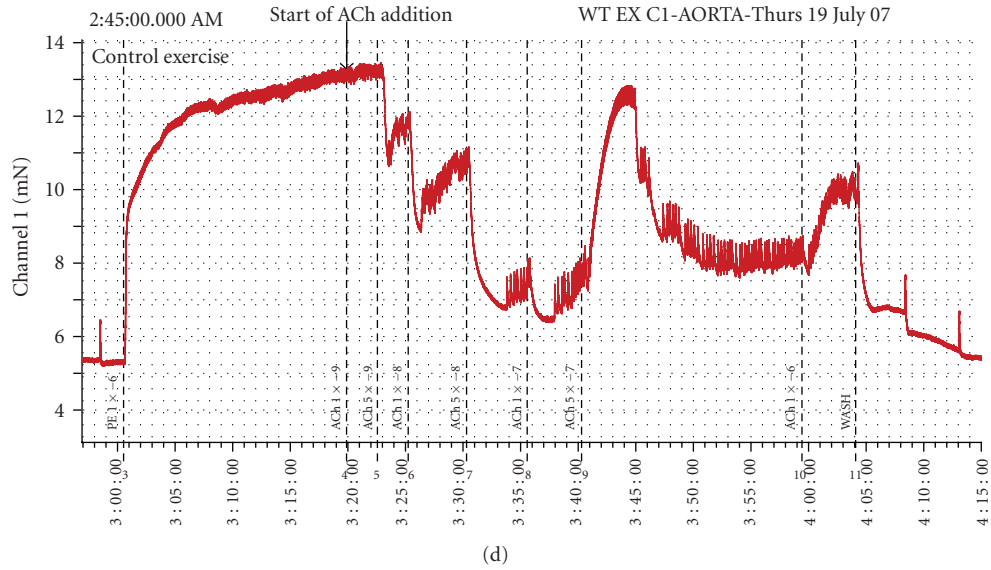

Figure 1: Representative traces showing ACh-induced vasodilation in aortae from diabetic (db/db) and control mice that were either sedentary or exercised.

Table 1: Body weights, plasma glucose CRP, and TNF-α levels in control or diabetic (db/db) mice that were either sedentary or exercised.

	Control sedentary			Control exercise		db/db Sedentary			db/db exercise	
	6 wk	10 wk	14 wk	10 wk	14 wk	6 wk	10 wk	14 wk	10 wk	14 wk
Body weight (gm)	20.7 ± 0.3	28.0 ± 0.3	31.9 ± 0.4	25.8 ± 0.4	28.3 ± 0.6*	30.7 ± 0.4#	45.9 ± 0.7#	48.9 ± 1.2#	43.6 ± 1.0#	44.6 ± 1.3*#
Fasting blood glucose (mg/dL)	2.3 ± 0.1	5.0 ± 0.2	5.9 ± 0.5	5.3 ± 0.2	5.8 ± 0.2	5.7 ± 0.3#	31.5 ± 1.3#	54.7 ± 1.5#	30.3 ± 1.9#	44.6 ± 1.6*#
Plasma CRP (ng/mL)	1.8 ± 0.3	2.6 ± 0.3	2.5 ± 0.4	3.8 ± 0.2*	4.5 ± 0.3*	3.3 ± 0.3#	4.1 ± 0.2#	5.1 ± 0.3#	3.8 ± 0.4#	3.6 ± 0.4*
PlasmaTNF-α (pg/mL)	N/A	N/A	14.88 ± 0.35	N/A	14.30 ± 0.74	N/A	N/A	18.62 ± 2.11	N/A	20.53 ± 1.85

*$P < .05$ compared to sedentary group at the same age.
#$P < .05$ compared to control groups.
N/A: variable not measured

4. Discussion

This study examined the effects of moderate levels of exercise on vascular endothelial function and plasma CRP levels in control and type 2 diabetic (db/db) mice. We report that endothelial function (endothelium-dependent relaxation) was significantly impaired in db/db mice, as also reported in other studies [17–19]. There is much evidence to support the notion that endothelial dysfunction precedes the development of type 2 diabetes [20, 21]. Two-week and six-week of moderate-intensity exercise both significantly improved endothelium-dependent relaxation in db/db mice.

There is a strong association between endothelial dysfunction and inflammation. Endothelial dysfunction and plasma markers of inflammation are consistently increased in type 2 diabetes [22]. Our results show that diabetic mice initially have higher CRP levels compared to control animals. An association between CRP levels and diabetes has been reported in other studies. For example, plasma levels of plasma CRP and ICAM levels are higher in diabetic subjects [23–25], and it is likely that increases in CRP levels also occur in patients with impaired glucose tolerance [26]. Thus, hyperglycemia may be one reason for endothelial dysfunction and low-grade inflammation in db/db mice [27]. Hyperglycemia is thought to activate the immune and macrophage-monocyte systems and so stimulate the production of cytokines and acute phase proteins, which are also proposed to reduce endothelial dependent vasodilation [22, 28]. Moreover, highly-glycated haemoglobin impairs NO-mediated vascular responses by a mechanism involving superoxide anions but not cyclooxygenase derivatives [7, 29]. In addition, db/db mice are obese, and there is also a close association between adiposity and CRP [13, 30]. Adipose tissue secretes inflammatory mediators (especially

Table 2: Citrate synthase activity (umole/mL/min) in thigh adductor muscle of all experimental groups.

	Control sedentary	Control exercised	db/db sedentary	db/db exercised
(10 Week old)	4.7 ± 0.053	5.0 ± 0.064*	3.6 ± 0.058	3.9 ± 0.042*
(14 Week old)	4.21 ± 0.32	6.61 ± 0.54*	1.67 ± 0.18	2.16 ± 0.12*

*$P < .05$ compared to sedentary group.

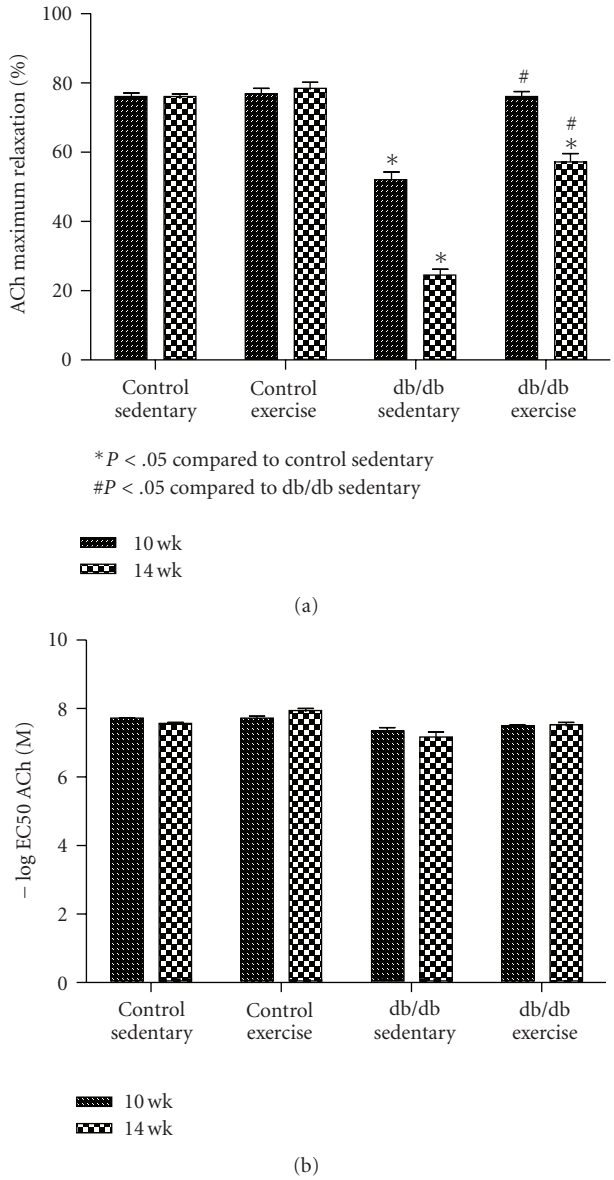

Figure 2: Emax (a) and EC50 (b) of ACh vasodilation after two weeks (10-week old mice) and six weeks (14-week old mice) of moderate-intensity exercise.

IL-6) which stimulates CRP synthesis in the liver [31]. CRP is related to insulin resistance and is a marker of endothelial dysfunction [32].

In our experiments, exercise improved endothelium-dependent relaxation in db/db mice after two-week exercise independently of reductions in weight, blood glucose, or plasma CRP levels; our data shows a lack of a correlation between improved vasodilatation to ACh and decreased plasma CRP levels after two weeks of exercise as shown by the nonsignificant ($P = .1941$) Pearson correlation coefficient for the relationship between maximal ACh dilation and plasma CRP levels. However, six weeks of exercise improved ACh-mediated vasodilatation while also reducing plasma CRP levels in db/db mice; this was associated with a significant correlation between plasma CRP levels and body weight, a finding that is consistent with other reports in experimental [33] and human diabetes [34].

Our results indicate that CRP levels are increased in control mice that underwent a period of forced-exercise. This finding is in keeping with recent studies in healthy humans indicating that there were significant increases in plasma CRP and TNF alpha following a two-week bout of exercise [35]. In addition, exercise has also been shown to stimulate a marked but transient increases in inflammatory markers such as IL-6 and cortisol (which subsequently stimulate hepatocytes to generate the synthesis of acute phase proteins such as CRP), responses that may reflect muscle injury [36, 37].

Since CRP can a cause dose-dependent decrease in NO production in endothelial cells [38], it is possible that this effect is time-dependent and occurs independently of inflammation as reported by CRP levels. Other studies have reported that that CRP directly inhibits the endothelium-dependent NO mediated dilation of porcine retinal arterioles [39], and down-regulates eNOS protein to decrease NO release [40].

The plasma levels of TNF-α in sedentary db/db mice tends to be higher than in control mice; however, it does not reach statistical significance. Previous reports have failed to demonstrate a parallelism between changes in plasma levels of CRP, IL-6, and TNF-α under pathological conditions [41–43]. Overweight adolescent boys had higher TNF-α, but not CRP or IL-6 levels compared to normal weight controls [42]. A systematic review demonstrated that exercise decreases CRP with no apparent effects on TNF-α [41]. However, CRP is the marker of chronic inflammation most frequently studied [44] and has been shown to predict cardiovascular diseases more than other cytokines [45].

In conclusion, we report a reciprocal association between endothelial dysfunction and CRP levels in diabetic db/db mice. Short-term exercise improves endothelial function without changing plasma CRP levels (two weeks of exercise). Longer periods of exercise (six weeks) reduce plasma CRP levels and maintain improved endothelial function in diabetic mice.

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Review Article

Inflammation, a Link between Obesity and Cardiovascular Disease

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Obesity, the most common nutritional disorder in industrialized countries, is associated with an increased mortality and morbidity of cardiovascular disease (CVD). Obesity is primarily considered to be a disorder of energy balance, and it has recently been suggested that some forms of obesity are associated with chronic low-grade inflammation. The present paper focuses on the current status of our knowledge regarding chronic inflammation, a link between obesity and CVDs, including heart diseases, vascular disease and atherosclerosis. The paper discusses the methods of body fat evaluation in humans, the endocrinology and distribution of adipose tissue in the genders, the pathophysiology of obesity, the relationship among obesity, inflammation, and CVD, and the adipose tissue-derived cytokines known to affect inflammation. Due to space limitations, this paper focuses on C-reactive protein, serum amyloid A, leptin, adiponectin, resistin, visfatin, chemerin, omentin, vaspin, apelin, and retinol binding protein 4 as adipokines.

1. Introduction

Obesity, the most common nutritional disorder in industrialized countries, is associated with an increased mortality and morbidity of cardiovascular disease (CVD) [1]. The World Health Organization estimates that more than 1 billion adults worldwide are overweight, 300 million of whom are clinically obese—defined as having a body mass index (BMI) equal to or greater than $30\,\mathrm{kg\,m^{-2}}$, or a waist circumference greater than 94 cm for men and 80 cm for women [2]. Obesity is a chronic, multifactorial, and complex disease resulting from a long-term positive energy balance, in which both genetic and environmental factors are involved [3, 4]. It was recently suggested that some forms of obesity are associated with chronic low-grade inflammation [5].

CVDs, including heart disease, vascular disease and atherosclerosis, are the most critical global health threat, contributing to more than one-third of the global morbidity. In most cases, these clinical conditions result from atherosclerosis, which was once identified as a lipid-storage disease. At the present time, CVD is recognized as a chronic inflammatory condition of the vessel wall that results from the transendothelial passage (transcytosis) of cholesterol-rich atherogenic Apo-B lipoproteins (VLDL, IDL and LDL) from the plasma into the intima. These lipoproteins are retained in the subendothelial space, which leads to infiltration of macrophages and T cells that ultimately then interact with each other and with the cells of the arterial wall [6, 7]. It is likely that inflammation induced by obesity accelerates the atherosclerosis. Adipose tissue is recognized as an important player in obesity-mediated CVD. In 1994, adipose tissue was first identified as the source of the hormone leptin, opening the door for a new era of research that focused on adipocyte endocrinology [8]. It is now apparent that adipocytes have a more complex physiological role [9]. Adipocytes produce large numbers of hormones, peptides, and other molecules that affect cardiovascular function, not only in an endocrine manner, but also by autocrine and paracrine mechanisms [10]. This might lead to cytokine-mediated inflammatory, changes in the liver, systemic inflammation and atherosclerosis.

This paper focuses on the inflammation related to obesity and CVD. It will discuss the methods of body fat evaluation in humans, the endocrinology and distribution of adipose tissue in the genders, the pathophysiology of obesity, the relationship among obesity, inflammation and CVD, and the adipose tissue-derived cytokines known to affect inflammation.

2. Methods of Evaluation of Body Fat in Humans

There are many methods that can be used to evaluate body fat in different populations [2, 11, 12]. While anthropometric measurements of weight-for-height have been traditionally used to evaluate obesity, more recently, BMI has become a standard parameter. BMI is defined as weight in kilograms divided by height in square meters. The normal range is 19–24.9 kg/m^2, with overweight defined as 25–29.9 kg/m^2, and obesity as $\geq$30 kg/m^2. BMI is not always a reliable measurement of body composition in individuals, particularly in older and younger people. Unfortunately, BMI does not provide any insight into regional body fat distribution. Thus, simple anthropometric measurements, such as waist circumference, can also be used to determine the valid index of visceral fat accumulation, in addition to being able to serve as an indicator of health risks associated with visceral obesity. A waist circumference of greater than 102 cm in men and 88 cm in women is a risk factor for CVD. A particularly important anthropometric parameter that has been increasingly applied in recent years is sagittal abdominal diameter (SAD) [13]. Using a simple caliper that was originally developed by Kahn, this anthropometric indicator can measure visceral fat tissue alone [14].

With regard to other techniques, one of the first that should be considered is the measuring of body density, as this provides information on the relationship between the body mass and volume. With tetrapolar bioelectric impedance analysis, data is obtained by measuring the resistance of the body exposed to the impact of an alternating current of 50 kHz at a strength of 800 μA. Radioisotopic techniques use deuterium or tritium as markers to measure the total body liquid and total body potassium. Infrared spectrometry is a simple but not particularly reliable method, based on the application of two sources of monochromatic light. Ultrasonographic measuring of fat tissue is currently the favored technique by which one can measure both the subcutaneous and visceral fat tissues. Measurements are carried out using a 7.5- and 3.5-mHz transducer for the subcutaneous and visceral fat tissue, respectively. The most accurate method for measuring central obesity is through the use of magnetic resonance imaging or computer-assisted tomographic scanning. Unfortunately, these approaches are too expensive for routine use.

3. Endocrinology and Distribution of Adipose Tissue between Genders

It is now apparent that adipose tissue is not simply a storage reservoir of fat, but is an active endocrine organ that plays multiple roles in the body. Adipose tissue contributes to the inflammatory process in obese subjects in both vascular and nonvascular tissues [15]. Abnormal levels of metabolites, such as lipids, fatty acids and, cytokines from adipose tissue, activate monocytes and increase the secretion of inflammatory cytokines. Adipose tissue from obese individuals contains activated macrophages that together with adipocytes produce various cytokines (Figure 1). These include inflammation-related adipokines such as leptin, adiponectin, tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), procoagulant substances such as PAI-1, vasoactive substances such as leptin, angiotensinogen and endothelin, and molecules that may contribute to insulin resistance such FFA, TNF-α and resistin. IL-1 signaling involves the type I Interleukin 1 receptor (IL-1R/IL-1R1), a Toll-like receptor that heterodimerizes with the IL-1R accessory protein (IL-1RAcP). Interleukin 1 receptor antagonist (IL-1Ra) is an anti-inflammatory cytokine that binds to IL-1R in competition with the proinflammatory cytokine IL-1. The relative occupancy of the IL-1R1-IL-1RAcP receptor complex with IL-1 agonist or with IL-1Ra determines whether the inflammatory signaling is "on" or "silenced", respectively. IL-1β induction of IL-6 and prostaglandin E2 (PGE2) signaling is indicated in Figure 3. In obesity, these cytokines are released into the circulation by adipose tissue, stimulating hepatic CRP production. Levels of the prothrombotic molecule PAI-1 are also increased, whereas adiponectin, which is produced exclusively by adipocytes, is decreased in obesity. One of the key vasoactive substances produced by adipocytes is leptin, which is an important regulator of food intake. Other adipocyte-derived molecules, including prostaglandins, adiponectin, and the more recently discovered resistin, affect metabolic function and might play a role in causing cardiovascular end-organ damage.

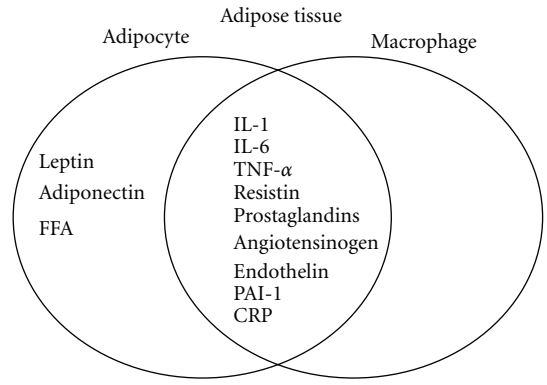

Figure 1: Cytokines secreted by adipocytes and/or macrophages in human adipose tissue.

Serum adipokine levels are elevated in humans and animals with excess adiposity. Visceral fat appears to produce several adipokines more actively than subcutaneous adipose tissue, and an increased abdominal adiposity in the visceral depot renders these individuals more prone to metabolic and cardiovascular problems [16]. Health

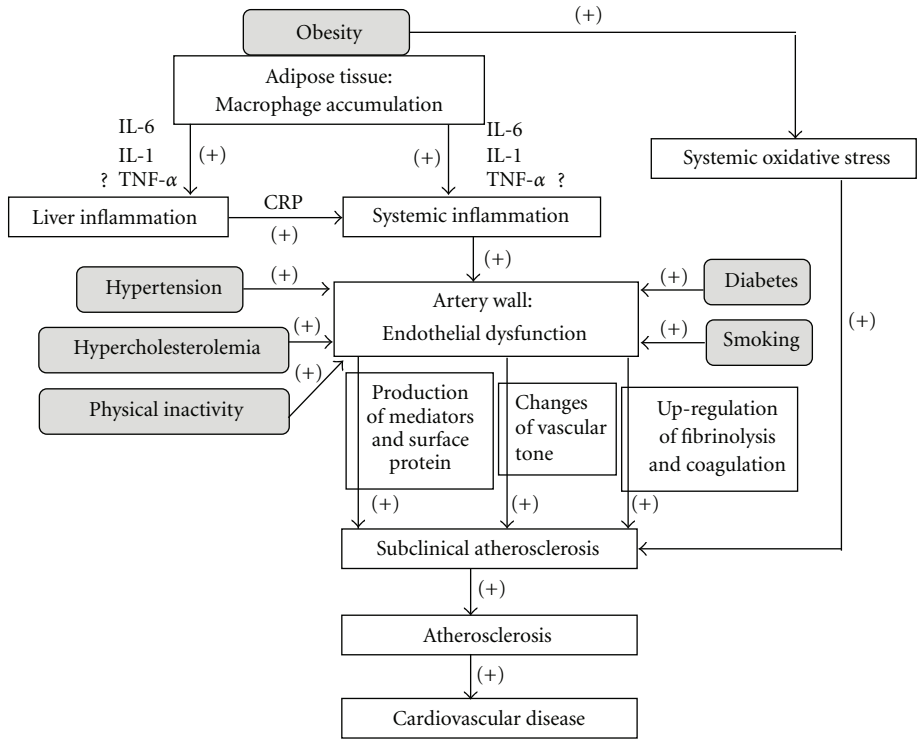

Figure 2: Mechanism of the relationship between inflammation induced by obesity and cardiovascular disease. Gray box shows the traditional cardiovascular disease (CVD) risk factors. The arrow and plus symbols indicate the enhanced courses. Smoking, obesity, hypertension, diabetes, physical inactivity and hypercholesterolemia are established risk factors of CVD. In obese individuals, macrophages first accumulate within the adipose tissue, leading to local inflammation. As the obesity increases, several proinflammatory factors, including IL-1, IL-6 and TNF-α, are produced in the adipose tissue. Macrophage accumulation and the subsequent local inflammation are believed to result in numerous metabolic dysfunctions that typically accompany obesity, including systemic inflammation. Endothelial dysfunction occurs during the early stages of atherosclerosis and is responsible for the pathophysiological changes in subclinical atherosclerosis, which include changes in a variety of mediators, surface proteins, and in autacoids that are involved in vasomotion, coagulation and inflammation. Obesity also can increase systemic oxidative stress independently of blood glucose and diabetes. One of the major events of atherosclerosis is CVD.

problems associated with obesity are generally related more to the central (abdominal, visceral) distribution of fat rather than to the amount of fat, and the distribution of fat differs between males and females [17, 18]. Men exhibit a more central accumulation of fat, whereas women exhibit a more gluteal/femoral accumulation. The original definition of obesity for males (android type) and females (gynoid type) dates to the first clinical observations made by Vague in 1947. The greatest health risk is associated with fat distribution in the central or upper body (android) parts. Recent research [19, 20] has shown that sex hormones play an important role in obesity and that there are differences in the occurrence of insulin resistance and heart diseases that are dependent upon gender. Findings have indicated that both the total amount of fat that an individual carries and the distribution of that fat are important. At present, it is difficult to accurately measure fat in the body, and there is currently no simple method available for routine clinical use.

Epidemiological and clinical evidence strongly suggests a major role for sex steroid hormones in the regulation of adipose tissue distribution. Sex steroid hormones, such as estrogen, progesterone, and androgen, are involved in the metabolism, accumulation, and distribution of adipose tissues. Normal distribution of body fat occurs when sex steroid hormones are present. If a decrease in sex steroid hormones occurs, such as that seen during aging or gonadectomy, there is a greater tendency for obesity states, in addition to increases in major risk factors for CVD.

4. Mechanisms of the Relationship among Obesity, Inflammation, and CVD

4.1. Systemic Inflammation. As individuals become obese and their adipocytes enlarge, the adipose tissue undergoes molecular and cellular alterations that subsequently affect systemic metabolism (Figure 2). First, macrophages accumulate within adipose tissue, leading to local inflammation. Several proinflammatory factors are produced in adipose tissue as obesity increases. When compared to lean individuals, adipose tissue in obese individuals shows higher expression of proinflammatory proteins, including TNF-α and IL-6 [21, 22]. Macrophage numbers in adipose tissue also increase with obesity [23], apparently acting as scavengers

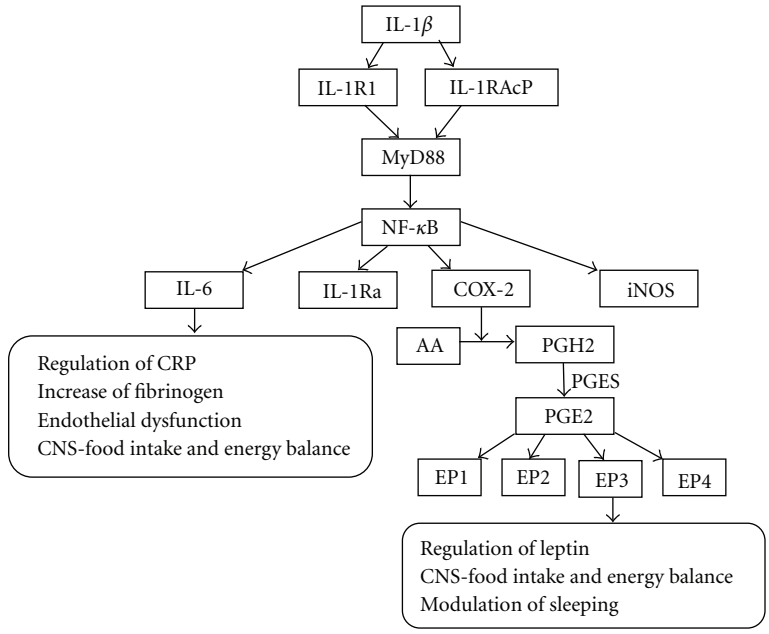

Figure 3: Interleukin-1β (IL-1β) induction of interleukin-6 (IL-6) and prostaglandin E2 (PGE2) signaling. IL-1β binds to the IL-1R1/IL-1R1AcP heterodimer, which then initiates the signaling cascade that causes the translocation of the transcription factor nuclear factor-κB (NF-κB) into the nucleus, where it induces the transcription of pro- and anti-inflammatory genes including inducible nitric oxide synthetase (iNOS), IL-6, IL-1Ra and cyclooxygenase-2 (COX-2). COX-2 catalyses the conversion of arachidonic acid (AA) to prostaglandin H2 (PGH2). PGH2 is converted into PGE2 by terminal PGE synthase (PGES). PGE2 signals occur via four different G-protein coupled receptors, EP1R-EP4R, each of which has multiple splice variants with different signaling properties.

of apoptotic adipocytes. It also has been reported that there is a marked increase in these scavengers in obese subjects [24]. Macrophage accumulation and the subsequent local inflammation are believed to result in numerous metabolic dysfunctions that accompany obesity, including systemic inflammation and atherosclerosis.

Visceral fat secretes more cytokines than subcutaneous adipose tissue [16]. A recent study elegantly demonstrated that transplantation of visceral adipose tissue from genetically obese mice into Apoe-deficient mice increased atherosclerosis in the recipient animals, suggesting that inflamed adipose tissue exerts distinct vascular effects, presumably through inflammatory cells such as macrophages within the visceral adipose tissue [25]. Macrophages within visceral adipose tissue are known to express and release cytokines. These cytokines reach the liver though the portal circulation, where they can stimulate hepatic inflammation [26], thereby inducing a chronic systemic inflammatory response.

4.2. Endothelial Dysfunction. Clinical and experimental data support a link between systemic inflammation and endothelial dysfunction. Mounting evidence shows that disturbed endothelial function may be an early marker of an ongoing atherosclerotic process. Thus, endothelial dysfunction has increasingly been recognized to play an important role in a number of conditions associated with a high prevalence of atherosclerotic CVD. Inflammatory cytokines are important protagonists in the formation of atherosclerotic plaques, eliciting effects throughout the atherosclerotic vessel. Importantly, the development of atherosclerotic lesions, regardless of risk factors (e.g., diabetes, hypertension, obesity), is characterized by the disruption of the normal function of endothelial cells.

The reasons for coronary endothelial dysfunction are complex and may involve ischemia/reperfusion injury. Smoking, obesity, hypertension, diabetes, physical inactivity, and hypercholesterolemia are established atherogenic risk factors. Endothelial dysfunction is regarded as an early stage of atherosclerosis, which is a chronic inflammatory disease [27]. Chronic inflammation is a major contributing factor to atherosclerosis and various markers of inflammation, fibrinolysis, and coagulation are upregulated in patients with established atherosclerotic disease. For vascular homeostasis, endothelial cells are of the utmost importance and they produce a variety of mediators, surface proteins, and autacoids involved in vasomotion, coagulation, and inflammation. Adipose tissue expresses enzymes involved in the angiotensin system (RAS) (renin, angiotensin-converting enzyme (ACE)), as well as the nonrenin-angiotensin system (NRAS) (cathepsin D, cathepsin G, tonin, chymase) [28]. The identification of elevated CRP as a transient independent risk factor for endothelial dysfunction might provide an important clue for linking a systemic marker of inflammation to the progression of atherosclerotic disease. Thus, CRP has been proposed for risk assessment of CVD in the at-risk general population. Available evidence suggests that low-grade inflammation is accompanied by decreased

bioavailability of endogenous NO and that TNF-α may play a key role in these events. The adipose tissue constitutes a source of other vasoactive factors, such as leptin, serum amyloid A (SAA), or apelin, among others [29]. Since blood vessels express receptors for most of the adipocyte-derived factors, adipose tissue seems to play a key role in cardiovascular physiology via the existence of a network of local and systemic signals. Therefore, these data demonstrate that markers of inflammation have independent predictive value for clinical and subclinical CVD beyond that of the traditional risk factors.

4.3. Subclinical Atherosclerosis and CVD. The development of atherosclerosis in obesity stems from a constellation of interrelated proatherogenic mechanisms. It is well established that a higher BMI is associated with subclinical inflammation, as reflected by increased CRP levels [30], and increased systemic oxidative stress that is independent of blood glucose and diabetes [31]. Recent evidence has suggested that leptin stimulates cholesterol uptake by macrophages, particularly in the presence of high glucose. This then triggers the formation of foam cells and the development of atheromatic lesions. Obesity-related hypoadiponectinemia might also contribute to impaired endothelial function, increased vascular ROS production and overall proatherogenic effects [32]. Finally, increased release of proinflammatory cytokines by adipose tissues, including IL-6, IL-1, and TNF-α, sustains vascular wall inflammation and promotes pro-atherogenic gene expression [33].

There is interest in identifying markers of subclinical atherosclerosis, such as coronary artery calcium (CAC) and carotid intimal medial thickness (CIMT), in order to facilitate an earlier diagnosis and possible prevention of CVD. CRP levels were found to be correlated with CIMT in a group of young subjects [34], but not in older individuals [35]. In other studies, levels of IL-6 have been shown to be associated with the amount of CAC [36], and the CD40 ligand, which is a marker of enhanced innate immunity, has been found to be correlated with CIMT in human subjects [37]. Since leptin levels have been shown to be associated with CAC independently of body weight measures or other risk factors, this points to a possible proatherogenic role for leptin [38].

5. Adipose Tissue-Derived Cytokines Known to Affect Inflammation

5.1. CRP. Of the many positive and negative acute-phase reactants, perhaps the most recognized is CRP, which is a member of the pentraxin family that attaches to the plasma membrane of damaged cells causing cell death through activation of the complement cascade [39]. More than 20 prospective epidemiologic studies have demonstrated that high-sensitivity CRP is an independent predictor of myocardial infarction, stroke, peripheral arterial disease, and sudden cardiac death, even in apparently healthy individuals [40, 41]. Clearly, CRP is one of the strongest markers of chronic inflammation, and it has been reported that it also directly participates in the coronary and aortic atherosclerosis that leads to cardiac events [42].

Ouchi et al. [43] confirmed CRP mRNA expression in human adipose tissue using quantitative real-time polymerase chain reaction. In the same article, the authors proposed that adipose tissue is an important source of circulating CRP. However, they made no attempt to investigate the stimuli able to induce CRP. Esposito et al. [44] investigated the effects of weight loss and lifestyle changes on vascular inflammatory markers in obese women. After 2 years, they found that BMI, as well as the serum concentrations of IL-6, IL-18, and CRP, decreased more in the intervention group than in the control subjects, whereas the adiponectin levels significantly increased. The beneficial effects of a Mediterranean-style diet on endothelial function and vascular inflammatory markers have been documented in patients with metabolic syndrome. When compared to patients consuming a control diet, patients consuming a Mediterranean-style diet have significantly lower serum concentrations of high-sensitivity CRP, IL-6, IL-7, and IL-18 as well as a decreased insulin resistance [45]. In a quartile analysis of the percent weight reduction, the largest weight reduction quartile did not show significant decreases in the CRP levels, whereas the middle quartiles showed remarkable CRP decreases. Based on inflammatory status, there may be an optimal pace of exercise combined with weight loss [46]. Two recent studies have demonstrated that exercise training in conjunction with weight reduction significantly affected the CRP levels, body composition, and human left ventricular growth [47, 48].

5.2. Serum Amyloid A (SAA). Serum amyloid A (SAA), an important marker of inflammation, is an apolipoprotein that is mainly synthesized in mammalian liver [49]. Human SAA is a 12.5-kDa protein whose levels can increase up to 1,000- fold in the serum 24–36 h after infection or injury, decline after 4–5 days, and then return to baseline after 10–14 days [50]. The human genome encompasses four SAA genes, of which three encode functional proteins. SAA1 and SAA2 are highly homologous reactants whose concentration can increase upon infection, trauma, and obesity [51, 52], whereas SAA3 is a pseudogene and SAA4 is a constitutively expressed minor constituent of the nonacute-phase HDL [53].

SAA has proven to be a suitable and sensitive indicator of the various stages of inflammation involved in inflammatory disorders. SAA is comparable to CRP, as both are major acute phase proteins that can increase up to 1,000-fold and reach 1 mg/mL in the serum under stimulation [50]. They can be produced by the liver under inflammatory stimuli, and their effects are mediated through pro-inflammatory cytokines (IL-1 and TNF-α) and "messenger" cytokines (IL-6) [54]. However, in contrast to CRP, which is mainly expressed in the human liver, SAA is expressed in both the liver and adipose tissue. SAA is now accepted as an adipokine that is produced by adipocytes and which directly mediates obesity-associated inflammation. Hence, SAA might serve as a better indicator of obesity and obesity-associated diseases,

especially when vascular diseases and metabolic disorders are present.

SAA is known to be a marker for obesity, as its expression is well correlated with obesity [55]. Some studies have shown that SAA levels are positively associated with BMI levels and that weight loss led to decreased SAA levels. In 1999, Danesh et al. [56] first reported that concentrations of SAA protein were strongly correlated with obesity. Since then, more than ten studies have shown that SAA is strongly associated with obesity [57–59]. In addition, it has been shown that SAA gene expression is increased in the adipose tissue of obese subjects and is significantly correlated with adipocyte size and inflammatory biomarkers [52].

Recent studies have shown that SAA elevation can predict cardiovascular events analogously with or even better than CRP by itself [60–62] and in this sense, it has been speculated that SAA might be one of the links or even a proatherogenic risk factor between inflammation and CVD [63, 64]. SAA is able to both alter vascular proteoglycans in a proatherogenic manner [65] and stimulate the production of various inflammatory mediators in cultured vascular endothelial cells, neutrophils, and monocytes [66]. Endothelial cells, smooth muscle cells, monocytes, and macrophages in atherosclerotic lesions have been reported to account for the extrahepatic production of SAA, as the presence of both SAA mRNA and protein products have been detected in these cell types [67]. SAA has also been accepted as being a biomarker of cerebrovascular disease and carotid artery intima-media thickness, which is an early index of atherosclerosis [68–71]. However, a very recent study indicated that SAA does not mediate early atherosclerosis [57].

SAA has also been found to be associated with metabolic disorders, such as diabetes, insulin resistance, and metabolic syndrome [72, 73]. Additionally, genes critical for insulin sensitivity were also found to be downregulated in adipocytes treated with recombinant SAA [74].

5.3. Leptin. Leptin, which was the first adipocyte hormone identified, influences food intake through direct effects on the hypothalamus [75]. The adipocyte-derived hormone leptin has actions in the brain (e.g., hypothalamus, cortex and limbic areas) and in a number of peripheral tissues as well (e.g., cells of the pancreas, liver and immune system). However, the central action of leptin in the brain, and in particular the hypothalamus, has been best characterized with regards to energy homeostasis and its importance for reproductive functions [76]. Moreover, disruption of peripheral leptin signaling in mice has been shown to cause no significant changes in either the energy balance or glucose homeostasis [77].

Mice lacking the gene coding for leptin (named *ob/ob* mice) are obese and diabetic. When *ob/ob* mice are treated with regular injections of leptin, they show reduced food intake, increased metabolic rate, and weight loss [78]. These effects appear to be mediated mainly by the central nervous system, as intracerebroventricular injection of leptin produces significant effects at much lower doses than those required by systemic injection. Systemic injections of leptin have a beneficial effect in children with congenital leptin deficiencies [79]. In a pioneering study, administration of exogenous leptin to individuals with lipoatrophic diabetes resulted in marked reductions in triacylglycerol concentrations, liver volume, and glycated hemoglobin. Ultimately, this treatment resulted in the discontinuation of or large reductions in the patient's antidiabetic therapy [80]. Unfortunately, leptin concentrations are already high in most obese individuals because of the increased amount of leptin-secreting adipose tissue [81]. In these individuals, increasing the leptin concentrations only induces the target cells to become resistant to actions of the hormone. Therefore, further studies need to be undertaken to clarify potential therapeutic strategies using leptin in these types of patients.

Leptin is involved in the control of not only energy homeostasis but also immunity. During fasting/starvation, when plasma leptin levels decline, neural pathways in the hypothalamus cause the appetite to increase and energy expenditure to decrease as the body attempts to restore its fat stores [82]. In addition, the fall in plasma leptin diminishes thyroid hormone production and inhibits the reproductive axis, both of which help to save energy during nutritionally lean times [83]. These metabolic effects of leptin are in part centrally mediated by activation of the hypothalamic-sympathetic nervous system axis [84]. In addition to the complete leptin deficiency disorder, relative leptin deficiency is an emerging clinical syndrome that is now being seen more often in several clinical conditions, including congential or acquired lipodystrophy as well as exercise-induced energy deficiency and hypothalamic amenorrhea or anorexia nervosa. Leptin replacement therapy might prove to be a therapeutic option for patients with these disorders [85]. Very recently, administration of chemical chaperones that decrease ER stress also restored leptin sensitivity in diet-induced obese mice [86]. In obese subjects who have lost weight, modifications that lead to decreased energy expenditure may predispose the individual to regain the weight. However, when subjects are administered "replacement" doses of leptin that restore their circulating leptin concentrations to preweight-loss levels, the weight gain can be prevented [87]. This suggests that the weight-reduced state is a condition of relative leptin deficiency. Recent reports have shown that in addition to its action on the hypothalamus, leptin may also act on the cortex and limbic areas, which are involved in cognitive and emotional regulation of feeding behavior [88]. Teleologically, the adaptations mediated by reduced leptin may have evolved as a protection against the threat of starvation by limiting energy use and enhancing energy storage [89].

The potential effects of leptin in the pathophysiology of cardiovascular complications of obesity remain diverse [90]. While some studies [91, 92] have indicated that circulating leptin levels are not significantly related to the risk of CVD or mortality in a diabetic population, these studies did find that leptin was associated with obesity and inflammatory markers. Even so, other reports have suggested that leptin does contribute to atherosclerosis and CVD in obese subjects

[93]. Therefore, this protein may elevate the blood pressure by stimulating the autonomic nervous system. Leptin has been found to have multiple effects on the cells of the artery wall. In human vascular endothelial cells, leptin upregulates the expression of the plasminogen activator inhibitor-1 [94], and leptin also helps modulate ACAT1 expression and cholesterol efflux from human macrophages [95]. In addition, leptin has been reported to increase nitric oxide (NO) bioavailability in blood vessels via the activation of endothelial NO synthase (eNOS) [96] and inducible NO synthase (iNOS) [97] in the endothelial and smooth muscle cells, respectively. Recent studies that measured coronary artery disease have demonstrated that hyperleptinemia was associated with coronary atherosclerosis [98, 99], with the association determined to be independent of insulin resistance. Other studies have shown that leptin may have a role in neointimal formation in response to arterial injury [100, 101]. In fact, very obese, leptin-deficient mice have been found to be protected from atherosclerosis despite all of the metabolic risk factors, suggesting that this hormone may directly contribute to the risk of vascular disease [102]. Moreover, in a prospective study in humans in which anthropometric and metabolic risk factors were controlled, the circulating leptin concentrations were shown to be an independent risk factor for predicting cardiovascular events [103]. Therefore, when chronically elevated concentrations of leptin are seen in obese individuals, this may indicate a predisposition to progression of atherosclerosis in these individuals.

5.4. Adiponectin. Adiponectin is a product of adipocytes, and its levels in humans decrease in obese subjects [104]. As one of the most extensively studied adipokines, adiponectin has 3 different oligomers, each of which may have a different biological function [105]. The major receptors for adiponectin are AdipoR1 and AdipoR2. These belong to a new family of receptors that are predicted to contain seven transmembrane domains but which will be structurally and functionally distinct from the G-protein coupled receptors. A recent study has shown that AdipoR2 stimulates energy dissipation by increasing fatty acid oxidation while inhibiting oxidative stress and inflammation [106, 107]. Adipocytes secrete high levels of adiponectin that then exert anti-inflammatory effects, most notably in atherosclerotic plaques [108]. These effects occur due to the suppression of TNF-α and proinflammatory cytokines such as IL-6 and interferon-c, along with the induction of other anti-inflammatory factors such as the IL-1 receptor antagonist [109]. In contrast, adiponectin levels have been shown to be low in several different forms of insulin resistance. In vivo, adiponectin enhances energy consumption and fatty acid oxidation in the liver and muscle, which reduces the tissue triglyceride content, thereby further enhancing the insulin sensitivity [110]. In adiponectin transgenic mice, there is improvement of the lipid profile [111, 112], and when plasma triglycerides are reduced, this leads to an increased VLDL catabolism in the skeletal muscle [113]. Taken together with its metabolic and anti-inflammatory effects, it has been proposed that adiponectin not only contributes to the beneficial effects of body weight loss but also has a role in modulating the cardiovascular system.

As might be expected based on the above observations, adiponectin promotes an antiatherogenic and anti-inflammatory program of gene expression and function in the vessel wall. Adiponectin downregulates the expression of adhesion molecules on the endothelial cells and directly improves endothelial dysfunction [114, 115]. Adiponectin also reduces proliferation in a receptor-independent fashion in the vascular smooth muscle cells [116]. In a very recent study, it has been shown that adiponectin reduces lipid accumulation, down-regulates the expression of scavenger receptors in macrophages, and promotes macrophage polarization, all of which play a role in anti-inflammatory activities [117]. Other studies have also indicated that adiponectin has an important role in cardiovascular protection. Hypoadiponectinemia is found in patients with angiographically demonstrated coronary artery disease [118]. In obese children, it has been reported that reduced adiponectin concentrations are of more importance than conventional cardiovascular risk factors, and that this inflammation status is related to early atherosclerosis [119]. However, in a large prospective study that was combined with a meta-analysis of previously published prospective studies, the adiponectin levels at baseline were found to be rather weak predictors of CVD risk [120]. However, other studies have shown that adiponectin exerts beneficial effects at nearly all stages of the atherogenesis process [121], and that the adiponectin levels are inversely correlated to the progression of the coronary artery calcium in both diabetic and nondiabetic subjects [122]. Serum total and high-molecular weight adiponectin are also associated with biomarkers of inflammation, insulin resistance, and endothelial function, all of which are independent risk factors of CVD [123].

5.5. Resistin. Resistin, which is one of the most recently identified adipokines, has been proposed to be an inflammatory marker for atherosclerosis. *While it has been shown to induce increases in CRP production by the macrophages [124], resistin is an example of the new adipokines that appear to have contrasting roles when examined in mice versus humans.* For example, confirmation of the results found in mice has proven to be difficult in human populations. This may be due to the fact that resistin appears to be derived from different sources in humans as compared to rodents. This protein was initially shown to be released in large amounts from mouse adipocytes, with obese mice having elevated levels that were accompanied by insulin resistance [125]. However, investigations in humans suggest that resistin is expressed in adipocytes with monocytes and macrophages [126, 127]. This lack of homology between the human and mouse resistin genes suggests a potential divergence in function [128]. Since macrophages are known inflammatory modulators, resistin may be an inflammatory marker in humans. Supporting this possible inflammatory role in humans are results that show recombinant resistin activates human endothelial cells, as measured by an increased expression of endothelin-1 and various adhesion

molecules and chemokines, while simultaneously increasing the CD40-ligand signaling by down-regulating the tumor necrosis factor receptor-associated factor-3 [129]. Moreover, Calabro et al. [130] has shown that resistin can promote human coronary artery smooth muscle cell proliferation by activation of the extracellular signal-regulated kinase 1/2 (ERK) and phosphatidylinositol 3-kinase (PI3 K) pathways. Taken together, these findings suggest a possible mechanistic link between resistin and cardiovascular disease via proinflammatory pathways.

In addition, there have been many recent reports that support a role for resistin in obese rodent models. Resistin has been found to modulate nutritional regulation and may possibly play a role in maintaining fasting blood glucose levels [131]. Further rodent studies have also suggested that resistin mRNA levels are higher in abdominal fat depots, as compared to deposits in the thigh [132], and that these serum resistin levels are positively correlated with BMI [133]. Recent investigations in humans have shown there are higher serum resistin levels in obese subjects as compared to lean subjects. These higher levels were also positively correlated with changes in the BMI and the visceral fat area [134, 135]. Lee et al. [136] found higher circulating resistin levels in obese mice when compared to their lean counterparts. Additional studies have reported significant reductions in circulating resistin levels following moderate weight loss [137] and postgastric bypass [138]. Collectively, these observations suggest that resistin may indirectly be involved in the nutritional regulation in humans.

5.6. Visfatin. Visfatin, also known as nicotinamide phosphoribosyltransferase (NAMPT), which was previously known as a pre-B cell colony-enhancing factor (PBEF), functions as a growth factor for early B cells within the immune system [139]. Fukuhara et al. [140] demonstrated that visfatin is a secreted protein that is expressed and regulated by the adipose tissue. As compared to subcutaneous adipose tissue, there are greater amounts of visfatin within visceral fat depots. Furthermore, this study indicated that visfatin could bind to and activate insulin receptors, similar to that seen for insulin both in vivo and in vitro. However, this effect of visfatin is controversial. For example, Revollo et al. were unable to reproduce the insulin-mimetic activity of this protein, even though a significant physiological role in the regulation of beta-cell function through the NAD biosynthetic activity was detected. Thus, the authors suggested that NAMPT could play an important role in the control of glucose metabolism [141]. After these novel findings, Fukuhara et al. decided to retract their previously published paper [142].

The visfatin peptide was originally discovered in the liver, skeletal muscle, and bone marrow and found to act as a growth factor for B-lymphocyte precursors. This peptide is not only produced by white adipose tissue (WAT), but also by endotoxin-challenged neutrophils, and is able to prevent apoptosis via a mechanism mediated by caspases 3 and 8 [143]. Circulating visfatin levels are closely correlated with WAT accumulation and visfatin mRNA levels increase in the course of adipocyte differentiation. Visfatin expression is up-regulated by IL-6 and TNF-α, and is down-regulated by GH [144]. Insulin has no effect on visfatin mRNA [145]. Moreover, visfatin is up-regulated by the peroxisomal proliferator-activated receptor (PPAR)-alpha and PPAR-gamma agonists in obese rats. Since it has been shown to be associated with improved glycemic control and lipid profiles, this suggests that PPAR-alpha and PPAR-gamma agonists may act, at least in part, via the up-regulation of visfatin expression [146]. In addition to inducing chemotaxis and the production of IL-1, TNF-α, IL-6, and costimulatory molecules by CD14C monocytes, visfatin also increases their ability to induce alloproliferative responses in lymphocytes, effects which are mediated intracellularly by p38 and MEK1 [144].

Possible associations between circulating visfatin and anthropometric or metabolic parameters in obesity and type 2 diabetes have been found in some but not all reported studies [147–149]. These contradictory findings may be due in part to the considerable differences found in the visfatin immunoassays [150]. In human studies, it has been shown there is a positive correlation between the visceral adipose tissue visfatin gene expression and BMI, along with a negative correlation between BMI and subcutaneous fat visfatin [151]. This suggests that visfatin regulation varies within different depots and that the adipose depot ratios are highly dependent upon the obesity of the subjects. A wide population study in humans has recently discovered a direct correlation between plasma visfatin and the BMI and body fat content in males only. This study failed to find any differences in the expression between the visceral and subcutaneous fat depots [152].

Several studies have shown that there are different disorders that exhibit altered plasma levels of this protein [153–156]. Thus, visfatin plasma concentrations may potentially be related to lipid metabolism [157] and the inflammatory response [158]. Since an increased expression of this protein has been observed in the macrophages of unstable carotid and coronary atherosclerosis in humans [159], and there is a negative association between the visfatin plasma levels of visfatin and vascular endothelial function [160], it has been proposed that visfatin plays a role in plaque destabilization. NAMPT, which was originally identified as PBEF, has been shown to act as a cytokine independent of its enzymatic activity, and thus plays a major part in regulating immune responses [161]. Since NAMPT has been implicated in the pathogenesis of several acute or chronic inflammatory conditions, such as atherosclerosis and CVD [161], it may act as a pro-inflammatory cytokine and potentially have a beneficial effect on insulin secretion.

At the present time, the role of visfatin in the modulation of glucose metabolism, as well as its ability to bind to the insulin receptor is still under debate [162–164]. As a number of inconsistencies among the different visfatin studies exist, the role of this adipokine in obesity and insulin resistance has yet to be clearly defined.

5.7. Chemerin. Recently, chemerin (retinoic acid receptor responder 2, tazarotene-induced gene 2) was found to be

highly expressed in adipose tissue and liver [165]. Chemerin is an agonist of the orphan G-protein coupled receptor chemokine-like receptor 1 (CMKLR1, ChemR23) [166] that is expressed by cells of the innate immune system [167]. Therefore, chemerin might be further evidence of a link between obesity and inflammation. Chemerin is secreted as an inactive precursor, and then activated through proteolytic cleavage by serine proteases of the coagulation, fibrinolytic and inflammatory cascades. Chemerin appears to be a novel and promising adipokine, and in several recent studies, human chemerin plasma levels have been shown to have a significant association with the BMI, inflammation, and metabolic syndrome [168–170].

Platelets have been found to be a rich cellular source of chemerin. In some pathological conditions, chemerin is activated and then released, which leads to the elevation of blood chemerin levels [171]. Recent studies have shown that both adipocytes [172] and fibroblast cells [173] can produce chemerin. Chemerin has also been measured in a number of human inflammatory exudates, including ascitic fluids from human ovary cancer and liver cancer, as well as synovial fluids from arthritic patients [174]. Angiotensin-converting enzyme (ACE) may be responsible for the activation of prochemerin. If so, as has been shown in vitro, this effect should be able to be blocked by an ACE inhibitor such as captopril [175]. However, further studies will be necessary to clarify this potential mechanism in vivo. There is also growing evidence that the bioactivity of chemerin is closely regulated by proteolytic cleavage in the C-terminal region, which may control its maximal chemotactic or anti-inflammatory effects [176]. While the primary amino acid sequences indicate that chemerin is structurally distinct from the CXC and CC chemokines, it functions exactly like a chemokine and can induce leukocyte migration and intracellular calcium mobilization. Chemerin also exerts potent anti-inflammatory effects on activated macrophages, which express the chemerin receptor CMKLR1 (chemokine-like receptor-1) in a cysteine protease-dependent manner [177].

Chemerin is a newly described adipokine with effects on adipocyte differentiation and metabolism in vitro [165]. In rodents, there is conflicting data with regard to the association of chemerin with obesity and diabetes. While there is a decreased chemerin expression in the adipose tissue of db/db mice as compared with controls [178], chemerin expression is significantly higher in the adipose tissue of impaired glucose tolerant and diabetic Psammomys obesus as compared with normal glucose-tolerant sand rats [179]. It has also been demonstrated that chemerin or chemerin receptor knockdown impairs the differentiation of 3T3-L1 cells into adipocytes, reduces the expression of adipocyte genes involved in glucose and lipid homeostasis, including adiponectin and leptin, and alters the metabolic functions in mature adipocytes [170]. In humans, no significant differences were noted for the chemerin levels between subjects with type 2 diabetes and normal controls. However, in normal glucose-tolerant subjects, chemerin levels were significantly associated with BMI, triglycerides, and blood pressure [179]. Plasma chemerin levels in normal subjects are also significantly associated with BMI, circulating triglycerides, and blood pressure, suggesting a strong relationship of this protein with obesity-associated complications [179].

It is possible that visceral fat may potentially contribute to the chronic inflammation that is observed in obese individuals. However, only a few studies have investigated the adipokine concentrations in the portal circulation [180]. In order to be able to determine the physiological role of chemerin in the glucose metabolism, and to identify chemerin's target tissues as well as relevant signal transduction pathways, further studies will need to be undertaken.

5.8. Omentin, Apelin, Vaspin, and Retinol-Binding Protein 4 (RBP4). Omentin, which was originally referred to as intelectin and first found in the intestinal Paneth cells, has a predicted molecular weight of 33 kDa [181]. Omentin is a fat depot-specific secretory protein synthesized by the visceral stromal vascular cells, but not the adipocytes. It has also been found in the human lung, intestine, and heart [182] and is strongly expressed in the human ovaries and placenta [183]. This new adipokine is codified by two genes (1 and 2) and is highly and selectively expressed in the visceral adipose tissue. In obesity, omentin 1 plasma levels and the adipose tissue gene expression are decreased, and there is a positive correlation with the plasma adiponectin and high-density lipoprotein. These levels were negatively correlated with waist circumference, BMI, and insulin resistance [184, 185]. Administration of glucose and insulin to human omental adipose tissue explants resulted in a dose-dependent reduction of the omentin-1 expression. Furthermore, prolonged insulin-glucose infusion in healthy individuals resulted in significantly decreased plasma omentin-1 levels [186]. Recombinant omentin enhances the uptake of glucose in isolated adipocytes and dramatically increases the insulin induction of Akt/PKB phosphorylation [182]. However, further studies need to be undertaken, as the physiological role of omentin in glucose metabolism along with omentin's target tissues, receptor, and the relevant signal transduction pathways have yet to be determined.

Apelin is a bioactive peptide that is produced by adipocytes, vascular stromal cells, the heart, and the cardiovascular system [187]. In humans, both obesity and insulin significantly elevate the plasma levels of apelin and this peptide appears to act as a circulating and paracrine hormone [187]. The gene that encodes the apelin, receptor shares the greatest sequence identity with the angiotensin AT1 receptor [187]. In experimental animal models of heart failure, the cardiac apelin system is down-regulated by angiotensin II, while restoration is achieved after treatment with an angiotensin type 1 receptor blocker [188]. In the cardiovascular tissues of rats, apelin production is up-regulated by hypoxia [189] and ischemic cardiomyopathy [190], which perhaps may be a compensatory mechanism. In spontaneously hypertensive rats, exercise training has also been shown to up-regulate the apelin production [191]. Apelin has a positive hemodynamic effect, as it acts an inotrope in both normal and failing rat hearts and in isolated cardiomyocytes [192, 193]. Apelin may be able to

regulate insulin resistance by facilitating the expression of brown adipose tissue uncoupling proteins and by altering adiponectin levels [194]. Decreased plasma apelin levels have been observed in patients with lone atrial fibrillation [195] and chronic heart failure [196]. Cardiac resynchronization therapy has been used to treat these patients successfully, with increases in the apelin levels observed after initiation of the therapy [197].

Vaspin is a member of the serine protease inhibitor family. This adipocytokine has been isolated from the visceral adipose tissue of Otsuka Long-Evans Tokushima Fatty (OLETF) rats that are at an age when the body weight and hyperinsulinemia has peaked [198]. OLETF rats are commonly used as a model of human type 2 diabetes. This model also shares common components of the human metabolic syndrome, including abdominal obesity, insulin resistance, hypertension, and dyslipidemia [199]. Vaspin production decreased at the same time the diabetes worsened and body weight fell in the untreated OLETF rats. However, when the animals were treated with insulin or pioglitazone, serum vaspin levels were maintained [198]. This suggests that the up-regulation of vaspin may have a defensive action against insulin resistance. Human vaspin mRNA has been reported to be expressed in the visceral and subcutaneous adipose tissue. In addition, it has been shown to be regulated in a fat-depot specific manner, and to be associated with obesity and parameters of insulin resistance [200, 201]. It has also been reported that elevated vaspin serum concentrations are correlated with obesity and impaired insulin sensitivity, whereas type 2 diabetes appears to abrogate this correlation [202, 203]. Vaspin expression decreases in conjunction with a worsening of the diabetes and a body weight loss. These studies indicated that vaspin might play a causative role in the development of obesity and metabolic disorders or, at least, be a biomarker for these diseases. In order to clarify these potential mechanisms, further investigation using more sophisticated methods will need to be undertaken.

Using the adipose-specific Glut4 knockout (adipose-Glut4(−/−)) mice model, retinol-binding protein 4 (RBP4) has been identified as a highly expressed circulating adipokine that causes insulin resistance when it is overexpressed or injected into mice [204]. In the circulation, RBP4 is bound to transthyretin, which causes decreases in the RBP4 renal clearance. In ob/ob mice, there was a 4-fold increase in transthyretin plasma levels as compared to lean mice or diet-induced obese mice [205]. A large number of subsequent studies confirmed there was an association between increases in the circulating RBP4 levels and various aspects of adiposity [206], insulin resistance [207, 208], diabetes mellitus [209], and metabolic syndrome [210, 211]. However, there are also other studies that have been unable to establish these associations [212, 213]. The reason for this discrepancy may be explained in part by the different methods that were used to measure the RBP4 and the different populations employed in these various studies. In some very recent studies, it has been reported that increased plasma RBP4 levels are associated with inflammatory cardiomyopathy [214] and cerebral infarction [215]. Therefore, at the current time, whether RBP4 functions as an adipokine in humans and exerts metabolic effects on glucose metabolism remains uncertain. Further studies will need to be performed in order to clarify RBP4's exact role in humans.

6. Conclusions

The worldwide incidence of obesity has markedly increased during recent decades. Obesity and associated disorders now constitute a serious threat to the current and future health of all populations on earth. Obesity represents a major risk factor for diseases including CVD,,atherosclerosis and diabetes, in which inflammation acts as a major driver in the pathogenesis. Both adipocytes and macrophages within fat tissue secrete numerous cytokines that may contribute to the characteristic pathophysiological changes. By expanding our knowledge on inflammation and the link between obesity and CVD, this should make it possible to improve our understanding of the pathophysiology of obesity.

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Review Article

Chronic Inflammation in Obesity and the Metabolic Syndrome

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The increasing incidence of obesity and the metabolic syndrome is disturbing. The activation of inflammatory pathways, used normally as host defence response, reminds us of the seriousness of this condition. There is most probably more than one cause for the activation of inflammation, a timeline of events related to the deterioration of metabolic homeostasis presenting along variable routes. Apparently, metabolic overload evokes several stress reactions, such as oxidative, inflammatory, organelle, and cell hypertrophy stresses, generating vicious cycles that amplify each other leading to dysfunction. Adipocyte hypertrophy, through purely physical reasons, facilitates cell rupture, what will evoke an inflammatory reaction. Inability of adipose tissue development to engulf all incoming fat leads to fat deposition in other organs, mainly in the liver, with marked consequences on insulin resistance. The oxidative stress which accompanies feeding, particularly when there is an excessive ingestion of fat and/or other macronutrients without concomitant ingestion of antioxidant-rich foods/beverages, may contribute to the inflammatory markers attributed to obesity. Moreover, recent data on the microbiota and its interaction with food and with obesity brought new hypothetic mechanisms for the obesity/fat diet relationship with inflammation. Beyond these common confounders, other phenomena, for instance, psychological and/or circadian rhythm disturbances, may likewise contribute to the raise of oxidative/inflammatory status. The difficulty in the management of the obesity/metabolic syndrome pathologies is linked to their multifactorial nature where environmental, genetic, and psychosocial factors interact through highly complex networks.

1. Obesity and the Metabolic Syndrome

The incidence of the metabolic syndrome is high and increasing. Metabolic syndrome refers to a constellation of disturbances including glucose intolerance, central obesity, dyslipidemia (hypertriglyceridemia, elevated nonesterified fatty acids (NEFAs), and decreased high-density lipoprotein (HDL) cholesterol), and hypertension [1]. It can present in several forms, according to the combination of the different components of the syndrome, and it is well established that it increases the risk for the development of cardiovascular disease, type 2 diabetes, and cancer [2–4]. However, it is not yet clarified how it begins and how the different components are causally connected among them. Different study groups have drawn special attention to one or another component. For example, the American Association of Endocrinology does not consider obesity as a component and highlights the importance of insulin resistance to the syndrome [5]. As a matter of fact, obesity and the metabolic syndrome do not completely overlap, evidence being now clear as to the existence of "benign" obesity [6–10]. In this metabolically healthy obese phenotype plasma concentrations of adiponectin are high, in good agreement with the effect of adiponectin overexpression in ob/ob mice that results in expansion of fat mass and protection against metabolic comorbidities [7]. The initial definition of the World Health Organization considered insulin resistance a key feature of the metabolic syndrome [11, 12], while the more recent National Cholesterol Education Program (NECP):Adult Treatment Panel III (ATP III) definition adds equal weight to any of the components of the syndrome: glucose intolerance, obesity, hypertension, and dyslipidemia [13].

Welsh et al. investigated the causal direction of the relationship between adiposity and inflammation using a bidirectional Mendelian randomization approach and came

to the conclusion that greater adiposity conferred by fat mass and obesity-associated gene and melanocortin receptor 4 single nucleotide polymorphisms led to higher C-reactive protein (CRP) levels, with no evidence for any reverse pathway [14]. Although this interesting finding needs to be confirmed and extended to other inflammatory markers, it supports the emphasis we are giving to adipose tissue in metabolic syndrome-associated chronic inflammation [15]. Whatever its origin, be it or not obesity the main initiator, the chronic low-grade inflammatory condition that accompanies the metabolic syndrome has been implicated as a major player in both the installation of the syndrome and its associated pathophysiological consequences [16]. In good agreement with this interpretation of things, weight loss of obese patients is repeatedly verified to be associated with a decrease of inflammation biomarkers [17–21] accompanied by improvement of metabolic parameters, namely, insulin sensitivity [22–26].

2. The Inflammatory Response

Inflammation is a physiological response of the organism to harmful stimuli, be they physical, chemical, or biological. The response provided usually conducts to the reestablishment of homeostasis. It involves the coordinated action of many cell types and mediators, whose intervention depends on the nature of the initial stimulus and ensuing responses thereafter. The normal acute inflammatory response involves the delivery of plasma components and leucocytes to the site of insult and is initiated by tissue-resident macrophages and mast cells leading to a production of different types of inflammatory mediators (chemokines, cytokines, vasoactive amines, eicosanoids, and products of proteolytic cascades) [16]. The activated endothelium allows extravasation of neutrophils and soluble components to the tissue, where they become activated releasing toxic agents and proteolytic enzymes to the extracellular milieu [27]. If successful, the injurious agent is eliminated and inflammation resolution and tissue repair follow. This is achieved by switching the lipid mediators from proinflammatory (e.g., prostaglandins) to anti-inflammatory and pro-resolution ones (lipoxins, resolvins, and protectins) and by the action of tissue-resident and newly recruited macrophages [27]. Tissue leucocytes undergo apoptosis and are phagocytised by the macrophages that leave the inflamed site by lymphatic drainage [28]. The apoptosis of inflammatory cells is a nonphlogistic physiological process of removing dead cells and is essential for the resolution of inflammation [16] with the added advantage that after engulfing these apoptotic cells, macrophages acquire a phenotype conductive to resolution, releasing anti-inflammatory signals such as interleukin-(IL-) 10 and transforming growth factor β (TGFβ) [29]. However, if the neutralization and removal of the noxious stimuli or even if the clearance of apoptotic inflammatory cells from the inflamed tissue fail, the inflammatory process persists and a state of chronic inflammation or autoimmunity may arise with different agents being recruited, namely, T lymphocytes and the development of lymphoid infiltrates in the tissue [30]. It has recently been demonstrated that the chronic inflammatory condition associated with morbid obesity is characterized by a continuous activation of the innate immune system [31].

3. Inflammation in Obesity and the Metabolic Syndrome

The inflammatory state that accompanies the metabolic syndrome shows a quite peculiar presentation, as it is not accompanied by infection or sign of autoimmunity and no massive tissue injury seems to have taken place. Furthermore, the dimension of the inflammatory activation is not large and so it is often called "low-grade" chronic inflammation. Other researchers have attempted to name this inflammatory state as "metaflammation", meaning metabolically triggered inflammation [32], or "parainflammation" as a term to define an intermediate state between basal and inflammatory states [33]. Whatever the term used, the inflammatory process that characterizes the metabolic syndrome seems to have its own unique features, its mechanisms being far from fully understood [15].

Recent studies have been confirming the positive association between obesity indices and inflammatory markers, mainly CRP protein in women [34, 35], but also other inflammatory markers, both in women and men [31, 36].

Increasing incidence of the metabolic syndrome all over the world accompanies adoption of the so-called modern Western lifestyle [37, 38]. The main negative features of this lifestyle include stress (long-term and continuous, psychological), positive energy balance (excessive energy intake and low physical activity), low-quality food (both high fat and energy dense, and at the same time poor in micronutrients), and disruption of chronobiology. An acute disturbance in any of the physiological regulatory systems evokes reactions that tend to reestablish equilibrium. When the stimuli, even of moderate magnitude, tend to be repetitive or chronic, change and allostasis in one system impact on the other, and vicious cycles are created and reinforced [1].

A state of positive energetic balance, with fat accrual along time, demands plasticity of the adipose tissue, which includes formation of new adipocytes and also physical compliance of the anatomical spaces for adipose tissue expansion [39]. Otherwise two deleterious phenomena ensue, hypertrophic growth of adipocytes that will rupture more and more frequently and fat deposition in organs other than adipose tissue [40], mainly in the liver, with local (nonalcoholic fatty liver disease) and systemic (insulin resistance) consequences. It is well known that the lack of adipose tissue, as evident in lipodystrophy [41], may equally lead to the development of metabolic syndrome.

After a meal, fatty acids are taken up by adipocytes, and during fasting or increased expenditure periods, the fatty acids are released to the blood stream. This is usually achieved through the coordinated action of hormones, catecholamines and insulin being the chief regulators of this balance [42]. Not only is the adipocyte metabolism altered by these hormones, but these hormones also regulate blood flow into the adipose tissue through the regulation of vascular tone, such that there is increased blood supply when the

organ is metabolically active [43]. Inadequate blood supply in a growing adipose tissue results in reduced oxygenation that may also contribute to inflammation [44].

The homeostatic capacity of adipose tissue development, with adipocyte hyperplasia responding to fat accommodation needs, has its limits and may contribute, in people that are not able to develop benign obesity, to adipocyte hypertrophy and the inflammatory response. Obesity researchers aimed, for some time, to find and study antiadipogenic agents to combat/prevent obesity, but that concept is no longer defensible [45–48].

3.1. Adipocyte Dysfunction and Inflammation. An increase in circulating concentration of NEFA [42] reflects the inability of the adipose tissue to buffer the excess nutrient intake and is related to the dyslipidemic state that is typical of the metabolic syndrome. When overload becomes present, the liver increases the production of apo-B containing particles that carry triacylglycerols to the adipose tissue resulting in low-density lipoprotein (LDL) formation [49]. This occurs in visceral adipose tissue very efficiently and this depot is also more capable of releasing lipids in times of requirement. The subcutaneous adipose tissue has usually a much larger capacity to store lipids given its usually larger size. This may explain why subcutaneous adipose tissue appears to be protective in terms of metabolic syndrome and why men, who for genetic and hormonal reasons possess a smaller subcutaneous adipose tissue compartment, achieve earlier the limit of that depot and overuse the visceral depot. When the capacity of both locations is overwhelmed, the conversion of very low-density lipoprotein (VLDL) or similar particles is delayed and hypertriglyceridemia originates [42]. Furthermore, other tissues are used for lipid accumulation (e.g., liver, muscle, pancreas, and heart) [40]. As these organs are not able to store lipids without harm to their functions, lipotoxicity may be the result culminating, in the case of muscle, liver, and pancreas, in insulin resistance.

Although it has become evident that adipocytes are involved in innate immunity, only recently was the presence of toll-like receptors (TLRs) in these cells described. The two most widely studied TLRs are TLR2 and TLR4, which are activated by bacterial lipoproteins and by lipopolysaccharide (LPS), respectively [50]. The engagement of either receptor leads to translocation of nuclear factor κB (NFκB) to the nucleus. In addition to their early recognized role in immunity, a participation in the regulation of metabolism is also being attributed to TLRs [50]. It has been shown that these receptors may be activated by specific types of lipids. It had already been demonstrated that the fatty acid moiety of TLR ligands was essential for their activation [51] and this led to the investigation of its possible activation by different sorts of lipids. Thus, it was discovered that saturated fatty acids activate both TLR2 and TLR4 and, instead, unsaturated fatty acids inhibit TLR-mediated signalling and gene expression [16]. This was also demonstrated with diet-derived saturated fatty acids, which increased TLR-mediated expression of IL-6 and tumor necrosis factor α (TNF-α), whereas unsaturated fatty acids had no effect alone but inhibited the saturated fatty acid-induced increase in TNF-α expression [16]. TLR4 has been found in murine preadipocytes 3T3-L1 and both TLR2 and TLR4 have been shown to be present and functional in human subcutaneous adipocytes [16]. Activation of TLRs results in synthesis of proinflammatory factors such as TNF-α, IL-6, and chemokines [16]. Considering the implication of the increase in circulatory NEFA in adipose tissue dysfunction, it is very likely that the activation of TLRs takes place in hyperlipidemic states, resulting in amplified inflammation and contributing to the development or aggravation of the metabolic syndrome [50].

There is compelling evidence showing that exposure of adipocytes to several types of stressors (oxidative stress, inflammatory cytokines, and elevated concentrations of fatty acids) induces cellular responses mediated by cellular kinases, including mitogen-activated protein kinases (MAPK; p38MAPK, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase), inhibitor of NFκB kinase (IKK) β, mammalian target of rapamycin (mTOR), and various conventional and atypical protein kinases C (PKC). Some of these kinases involved in stress-sensing are related to the impairment of insulin action through the stimulation of insulin receptor substrate (IRS) serine phosphorylation but also often activate targets related to the inflammatory response [32]. The three main kinases that have been related to this inactivation of IRS are JNK, IKK and PKC [16]. They exert powerful effects on proinflammatory gene expression, through activation of activator protein-1 (AP-1) complexes and NFκB [52].

Metabolic dysfunction due to metabolic overload also seems to be mediated through IKKβ. The reduction of IKKβ expression partly protects mice from obesity-induced insulin resistance, and the inhibition of this kinase achieved by high doses of salicylates has been shown to improve insulin sensitivity in humans and other experimental models [16]. PKC has also been shown to constitute an important interface between metabolic deregulation, inflammation, and insulin resistance. This kinase (isoform θ in skeletal muscle and δ in the liver) can be activated by fatty acid metabolites that accumulate due to metabolic pathway burden such as fatty acyl CoA and diacylglycerol, leading to inhibitory serine phosphorylation of IRS and attenuation of insulin signalling [16]. Furthermore, PKCθ is known to activate IKK and might contribute to insulin resistance and amplification of inflammation.

The metabolic stress to which adipose tissue is subjected in obesity, together with other already mentioned stresses, results in organelle dysfunction, particularly in mitochondria and the endoplasmic reticulum (ER). The ER is a cytosolic organelle that participates in the regulation of lipid, glucose, cholesterol, and protein metabolism, apart from being the site of triacylglycerol droplet formation. In the obese, the adipocyte may be especially challenged, given that it is required to secrete large amounts of substances and synthesise lipids. Under such conditions, ER function may be impaired leading to the accumulation of misfolded or unfolded proteins in its lumen. As a way to cope with it, the stressed endoplasmic reticulum engages the unfolded protein response (UPR). The UPR functions via signalling through three branches, denoted for the three stress-sensing proteins

found in the ER membrane: PKR-like eukaryotic initiation factor 2a kinase (PERK), inositol-requiring enzyme-1 (IRE-1), and activating transcription factor-6 (ATF-6) [53]. Their activation attenuates the cellular workload (decreasing protein translation, clearance, and degradation of excess proteins from the ER lumen) and induces repair (induction of an antioxidant response and of chaperone transcription to assist with the unfolded proteins) and ER biogenesis, towards recovery and survival of the cell. However, if the ER stress is not relieved, the UPR may also induce cell death via apoptosis. JNK, after activation by IRE-1, is an important effector in this action and, apart from this role, it may lead to a variety of other downstream effects depending on the cellular context, such as cell survival, inflammation, and insulin resistance. The activation of inflammation by the UPR also depends upon the IKK-NFκB pathway, also through IRE-1α, resulting in increased TNF-α and IL-6 production, further supporting its contribution to insulin resistance [16].

Obesity is associated with deregulated lipid and carbohydrate metabolism. An increase in either one of these substrates will also increase the demand on the mitochondria and the utilization of the electron transport chain [54]. As in metabolically active tissues undergoing increased demand, there is usually relative hypoxia, together with the increased need for nutrient oxidation. This generates unusual amounts of reactive oxygen species. Oxidative stress activates kinases like JNK, p38 MAPK, and IKK that may directly interfere with insulin signalling or indirectly via induction of NFκB and increased cytokine production [16].

Furthermore, the enhanced flux through alternative metabolic pathways leads to the generation of other metabolites that may themselves be related to impairment of insulin function and inflammation. Examples include fatty acyl CoA, diacylglycerol, which activate PKC serine kinases, and ceramide, which can undergo phosphorylation to ceramide-1-phosphate and promote inflammation or originate secondary metabolites with the same effect. Ceramide may also activate protein phosphatase 2A, which inactivates PKB/Akt and attenuates insulin response, contributing to insulin resistance [55].

3.2. Visceral Obesity. Adipose tissue pathogenicity differs according to adipose tissue localization, visceral, or subcutaneous [56]. Visceral adiposity seems to be an independent predictor of insulin sensitivity [57–59], impaired glucose tolerance [60], elevated blood pressure [61, 62], and dyslipidemia [58, 63]. Visceral fat is a highly active tissue from the metabolic point of view. It is apparently more susceptible to lipolysis than subcutaneous adipose tissue [64] and is associated with higher production of TNF-α [64, 65], plasminogen activator inhibitor-1 (PAI-1) [66], IL-6, and CRP [67]. On the other hand, it is a feabler producer of adiponectin, an adipokine more strongly correlated with subcutaneous fat [68]. Bahceci and collaborators [69] found a positive correlation between adipocyte size and TNF-α, IL-6, and high-sensitivity CRP. On the other hand, adiponectin was found to be negatively correlated with adipocyte size.

Although visceral obesity seems to play a central role in the metabolic syndrome, being generally considered much more proinflammatory [70], not all patients with the syndrome present this feature. Perhaps more important than the amount of accumulated fat in the abdominal cavity is the size of abdominal adipocytes. We have shown that big adipocytes are more prone to rupture [45], and cell rupture will obviously constitute a focus of inflammation. Macrophage crowns surrounding dead adipocytes in adipose tissue, as shown by Cinti and collaborators [71], are compatible with this hypothesis.

As already stated, adipocytes behave as immune cells [72–75] and are able to synthesize and release a huge amount of proinflammatory adipokines and cytokines including leptin, resistin, PAI-1, IL-6, TNFα, retinol-binding protein 4 (see [16]), IL-1β, monocyte chemoattractant protein-1 (MCP-1), CRP, macrophage migration inhibitory factor (MIF) (see [76]), chemokines from the CC and CXC families [77], and more recently described cytokines such as IL-18 [78] and IL-33 [79], most of which, if not all, are involved in insulin resistance [76, 80]. But beyond the capacity of adipocytes to secrete these and possibly more proinflammatory cytokines, under circumstances of obesity and/or nutrient overload, adipose tissue macrophages do also provide their counterpart of insulin-resistance inducing cytokines. Moreover, obese adipose tissue do also contain lymphocytes that participate in and reinforce the inflammatory reaction and consequent insulin resistance [77, 81, 82].

Whereas subcutaneous adipocytes can also become hypertrophic, and most probably do also rupture, visceral adipocytes, besides being supported by much less dense connective tissue in comparison with subcutaneous adipose tissue, are frequently subject to sudden pressure variations associated with cough, physical exercises [83], and sleep apnea [84, 85]. Intra-abdominal pressure is higher in obese patients [86], what may also impact on adipocyte stability. Preadipocytes of upper-body obese women exhibit reduced differentiation and are more prone to apoptosis than preadipocytes isolated from adipose tissue of lower-body obese or lean women [87]. The factors involved in this preadipocyte behaviour deserve investigation [88]. Moreover, it has been proposed that visceral adipose tissue growth is mainly due to hypertrophy, while in other locations there may be mainly growth through hyperplasia [89]. The physical constrains presented inside the abdominal cavity may halt adipogenic differentiation of adipose tissue precursors reducing the number of competent cells to accumulate excessive energy ingested. As a matter of fact, it has been shown that stretching inhibits adipocyte differentiation [16].

In an interesting model of diet-induced metabolic syndrome, the fructose-fed rat, the animals exhibit a series of metabolic syndrome features but do not weigh more than controls. However, their abdominal adipocytes are hypertrophic, a modification prevented by blockade of the renin-angiotensin system [90]. Functional relationships between adipocytes and the renin-angiotensin-aldosterone system [91] or between adipocytes and adrenocortex/aldosterone [92] do probably deserve more attention. A critical role for the balance between gluco- and mineralocorticoid action

in determining adipocyte responses implicated in obesity-associated inflammation and cardiovascular complications has recently been demonstrated [93].

Another characteristic of abdominal adipose tissue is its high metabolic activity and dense vascularisation. This high vascularisation is most probably due to the action of angiogenic/proinflammatory factors, of which leptin constitutes an example [94, 95]. On the other hand, increased intra-abdominal pressure, as well as pressure variations, may easily create periods/zones of hypoxia, contributing to the production of hypoxia inducible factors. Vascular endothelial growth factor (VEGF), leptin, adenosine, and substance P, among others, will impact on other features of the metabolic syndrome. Furthermore, hypoxia by itself inactivates the adiponectin promoter [96].

3.3. Diet, Microbiota, and Inflammation. Among the complex interaction between genetic, metabolic, and environmental factors that is most probably associated with the present prevalence of obesity and metabolic syndrome, dietary patterns are considered of central importance. In these, attention has been focused over calories, amounts, and proportions of macronutrients, and their effects on the energetic balance by themselves, and through metabolic regulators. Only recently have the acute effects of food ingestion, taking into consideration the type of food, and the specific effects of some nutrients, namely, fatty acids, began to be studied in relation with obesity and inflammation.

Total dietary fat and saturated fat are associated with insulin resistance and high blood pressure as well as obesity-related inflammation [97]. An immediate postprandial increase in plasma inflammatory markers after a high-fat meal had been shown in abdominally obese men [98]. Consumption of a saturated fatty acid-rich diet resulted in a proinflammatory "obesity-linked" gene expression profile, whereas consumption of a monounsaturated fatty acid-rich diet caused a more anti-inflammatory profile [99]. Moreover, acute dietary n-3 PUFA dietary supplementation has been shown to improve fasting as well as postprandial lipid metabolism and components of the associated inflammatory response in the JCR:LA-cp rat [100]. Increased central and overall adiposity in children is associated with higher circulating adipocyte protein 2 (aP2) concentrations, high dietary intakes of total fat and saturated fat influencing aP2 association with insulin resistance and inflammation [101]. Intakes of total fat and antioxidant vitamins have been shown to be determinants of subclinical inflammation in the same children [102].

It must, however, be taken into account that inflammatory markers increase even after a mixed meal, more so in children with central obesity [103]. The proinflammatory effect of a high-fat diet per se is shown in a most interesting investigation by the group of Rankin: people put under a weight loss diet and that effectively lost weight increased the concentration of an inflammation marker when the diet was high-fat, differently from a low-fat high carbohydrate diet where the inflammatory marker decreased with the loss of weight. They also showed that the proinflammatory effect of the high-fat diet was not dependent on oxidative stress [104, 105]. These results are corroborated in a recently published experimental research work in mice, where it has been shown that a shift from a high-fat to a high-carbohydrate diet improved levels of adipokines and proinflammatory cytokines [106].

Intestinal microbiota, microorganisms which are known to contribute to digestion and metabolism, have barely been taken into consideration in this discussion, but for some recent papers [107–111]. However, the extension and metabolic gene *repertoire* of our microbiota are compatible with huge metabolic consequences for the host depending on the microbiota constitution.

Comparison of microbiota from obese and nonobese subjects has shown different proportions of certain species [112], but it remains unknown what is cause and consequence, or the metabolic consequences, for the host, of harboring different microbiota. This remains an almost virgin soil, in spite of the putative importance of conditioning the evolution of the microbiota in the metabolic interest of the host. Some associations between dietary patterns and resistance to obesity, namely, the importance of the diet acid load [113], nondigestible plant material [114], or the richness in certain minerals [115] may indeed include effects of those elements on the microbiota.

Cani and collaborators have demonstrated that high-fat feeding increases intestinal permeability and the plasma concentration of LPS, that is, metabolic endotoxemia, and that changes in gut microbiota control this metabolic endotoxemia and inflammation [116]. Therefore, high-fat feeding may have direct proinflammatory /anti-inflammatory effects, depending on the nature of fatty acids, long-term inflammatory consequences related with overload of adipose tissue, and indirectly modulate metabolic endotoxemia and inflammation through effects on the microbiota.

4. Conclusion

It has become evident that the inflammatory condition that is associated with obesity and overweight plays an important part in the aetiology of the metabolic syndrome and largely contributes to the related pathological outcomes. From the reasons here presented, it seems likely that adipocyte dysfunction lies at the bottom of this question, its homeostatic functions being overwhelmed due to metabolic overload. From then on, several vicious cycles exacerbate the disturbances and lead to an inflammatory response, most intense when adipocytes reach large dimensions and easily rupture for mechanical reasons. In parallel with this, the effects of high-fat diets, frequently consumed by obese subjects, add to the inflammatory fire, both directly, when the fat is rich in saturated fatty acids, and indirectly, through effects on the microbiota and intestinal permeability (Figure 1).

Progress in the knowledge of this highly complex network of pathophysiological events that include multiple cell types, cytokines, nutrients, in a variable nervous and hormonal status, as well as specific physical constraints and microbial colonization, will open the way for effective therapeutic interventions. Inflammatory mediators are

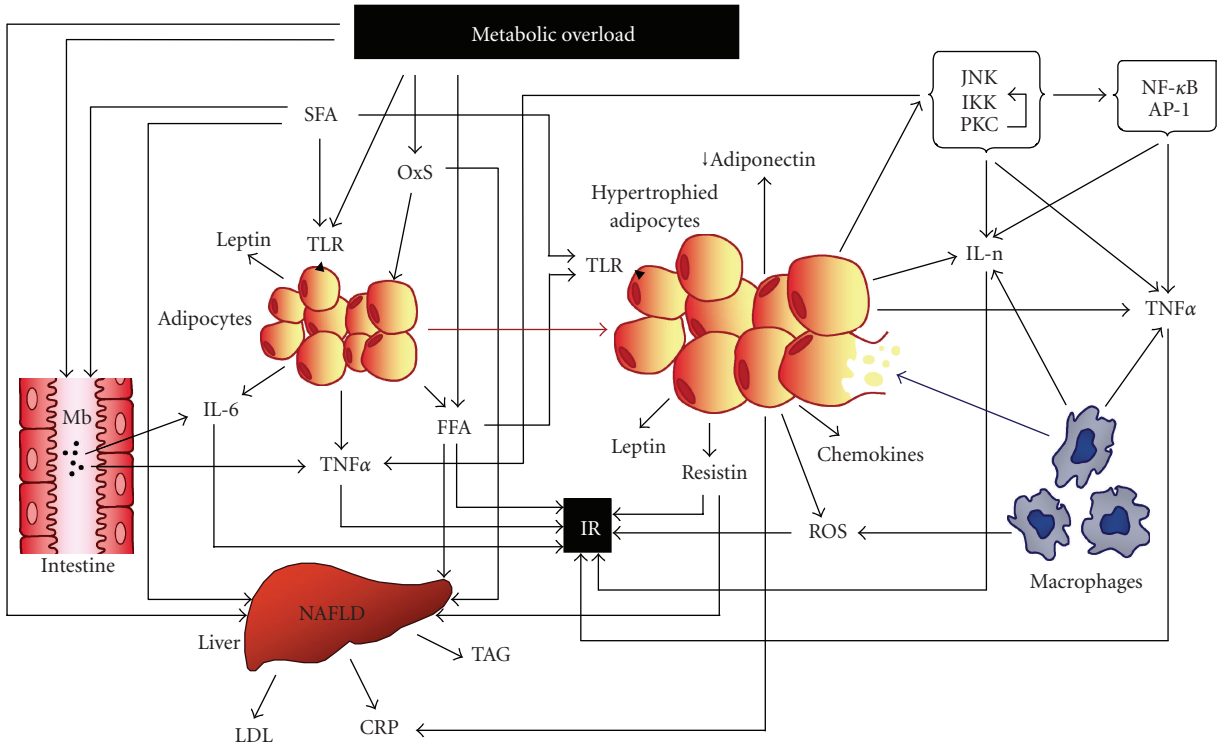

Figure 1: Overview of the complex interplay between obesity-inflammation-metabolic syndrome: metabolic overload impacts on adipose tissue, leading to organelle stress with production of ROS and adipokines, as well as activation of kinases that potentiate the transcription of inflammatory genes and interfere with insulin signaling. Hyperthrophy facilitates rupture of adipocytes which attract and activate macrophages that markedly reinforce the inflammatory process through further production of ROS and inflammatory cytokines. Production of adiponectin, an anti-inflammatory cytokine, is reduced. Increase of FFA concentration, namely, SFA, coming both from feeding and adipose tissue overflow, accumulates in the liver, among other organs. Fat accumulation in the liver leads to overproduction of LDLs and, together with IL-6, of CRP. NAFLD is a frequent consequence of these metabolic dysregulations, and all this impacts on insulin sensitivity. SFA activates TOLL-like receptors in adipocytes, contributing to the activation of the inflammatory response. Fat has also effects on intestinal permeability and on the microbiota, with systemic inflammatory consequences. Most excess metabolites and cytokines produced throughout these processes converge on insulin resistance, a central characteristic of the metabolic syndrome. AP-1: activator protein-1; CRP: C-reactive protein; FFA: free (nonesterified) fatty acids; IL-n: interleukins; IKK: inhibitor of NF-κB kinase; IL-6: interleukin-6; Int: intestine; IR: insulin resistance; JNK: c-Jun N-terminal kinase; LDL: low density lipoprotein; M: microbiota; NAFLD: nonalcoholic fatty liver disease; NF-κB: nuclear factor κB; OxS: oxidative stress; ROS: reactive oxygen species; PKC: protein kinase C; SFA: saturated fatty acids; TAG: triacylglycerols; TLR: TOLL-like receptors; TNFalpha: tumour necrosis factor alpha.

already showing their usefulness as biomarkers of the metabolic/inflammatory/disease-prone status of patients with the metabolic syndrome.

But diet will remain playing a crucial role in multiple aspects of this large picture. And, although much remains to be known in what respects nutrition, the biggest challenge will be to reinstall a nonobesogenic lifestyle.

List of Abbreviations

AP-1: Activator protein-1
aP2: Adipocyte protein 2
ATF-6: Activating transcription factor-6
ATP III: Adult treatment panel III
CoA: Coenzyme A
CRP: C-reactive protein
ER: Endoplasmic reticulum
HDL: High-density lipoprotein
IKK: Inhibitor of nuclear factor κB kinase
IL: Interleukin
IRE-1: Inositol-requiring enzyme-1
IRS: Insulin receptor substrate
JNK: C-Jun N-terminal kinase
LDL: Low-density lipoprotein
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MCP-1: Monocyte chemoattractant protein-1
MIF: Migration inhibitory factor
mTOR: Mammalian target of rapamycin
NECP: National cholesterol education program
NEFA: Nonesterified fatty acids
NFκB: Nuclear factor κB
PAI-1: Plasminogen activator inhibitor-1
PERK: PKR-like eukaryotic initiation factor 2a kinase
PKB: Protein kinase B

PKC: Protein kinase C
TGFβ: Transforming growth factor β
TLR: Toll-like receptor
TNF: Tumor necrosis factor
UPR: Unfolded protein response
VEGF: Vascular endothelial growth factor
VLDL: Very low-density lipoprotein.

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