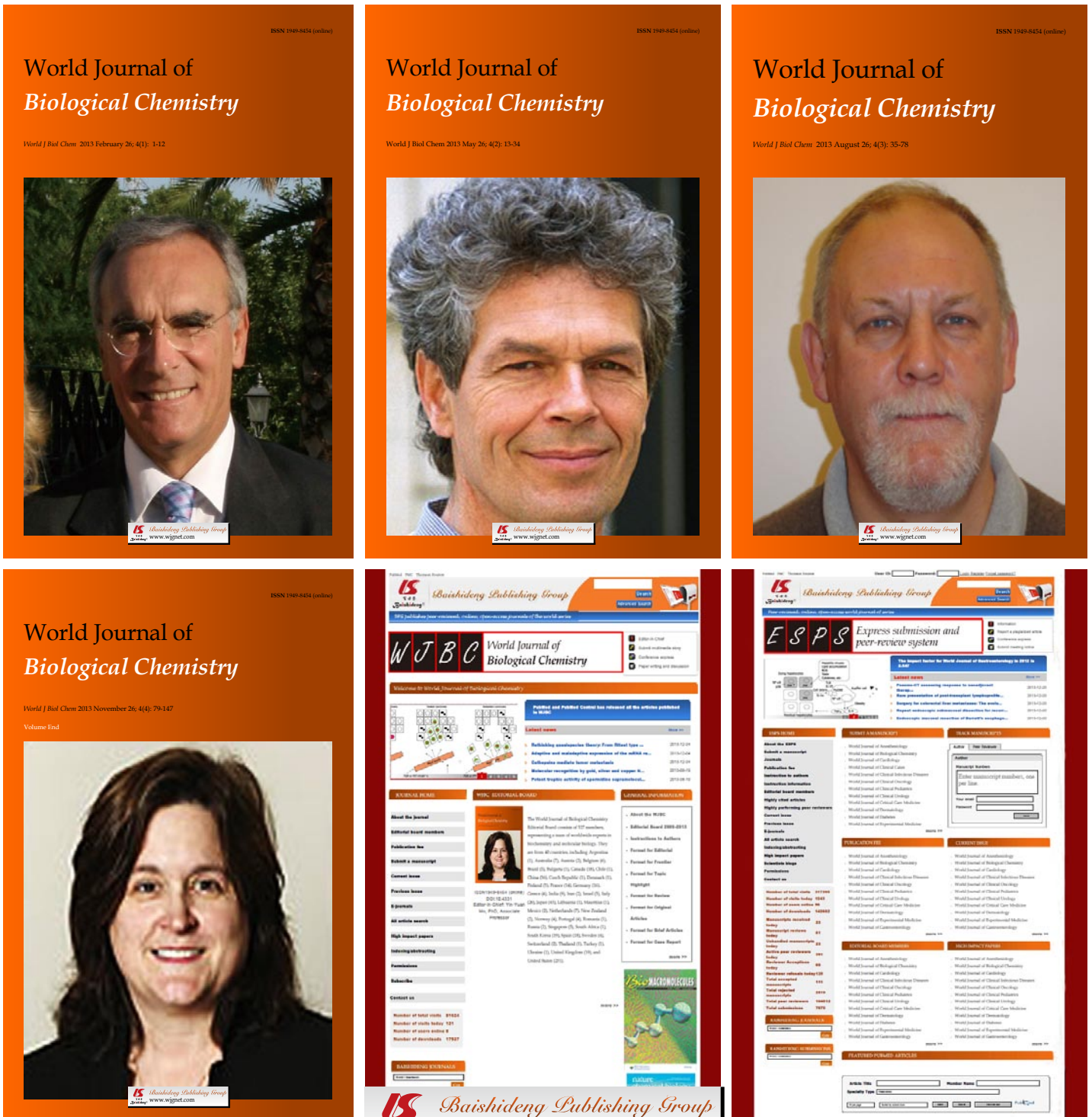
ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

2013 Bound Volume 4 Issue 1-4: 1-147



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World Journal of Biological Chemistry

A peer-reviewed, online, open-access journal of biological chemistry

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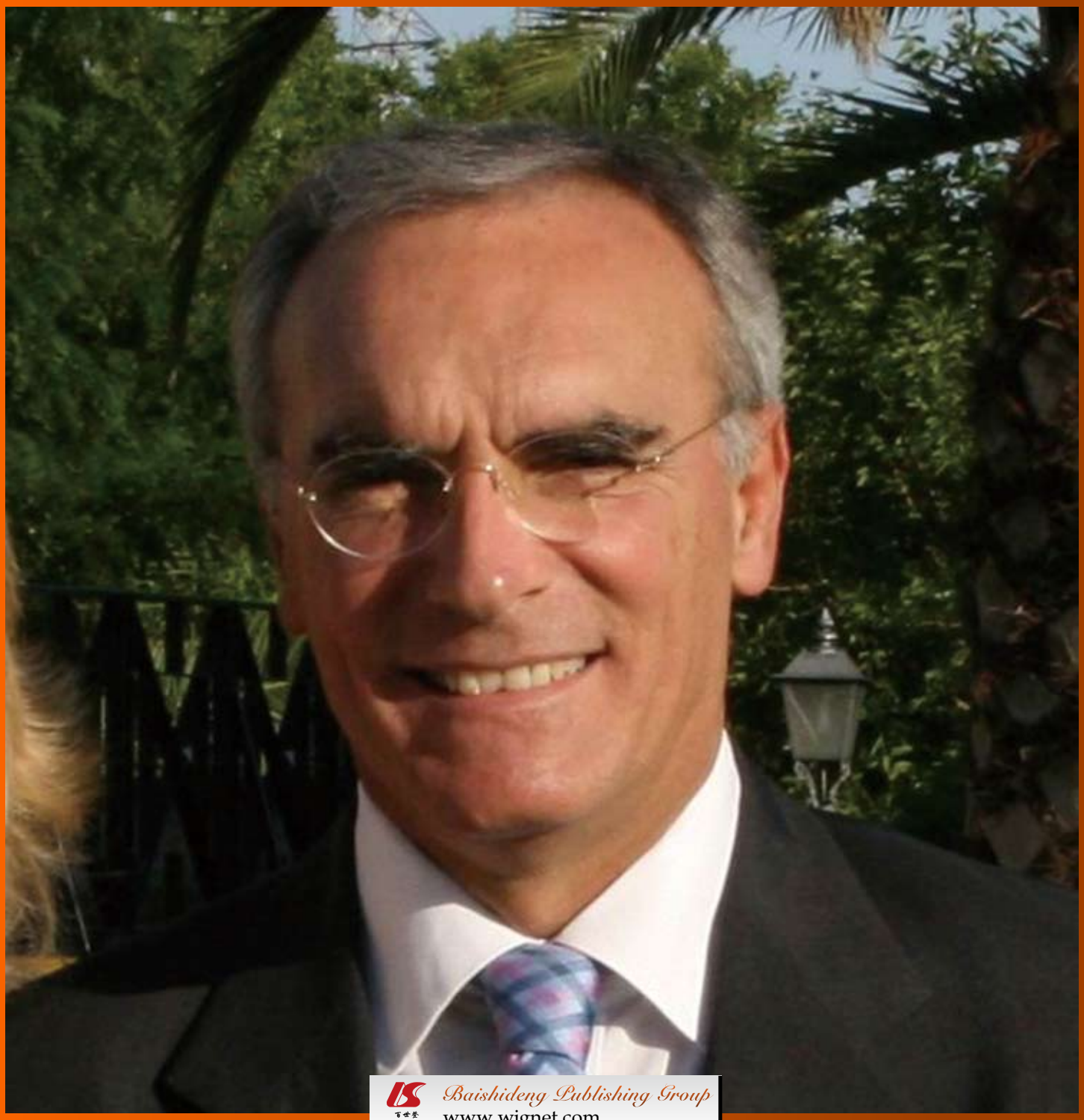
ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2013 February 26; 4(1): 1-12



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World Journal of Biological Chemistry

Contents

Quarterly Volume 4 Number 1 February 26, 2013

World Journal of Biological Chemistry
Volume 4 Number 1 February 26, 2013

Contents

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Quarterly

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PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F, Lucky Plaza,
315-321 Lockhart Road, Wan Chai,
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Telephone: +852-6555-7188
Fax: +852-3177-9906
E-mail: bpgoffice@wjgnet.com
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PUBLICATION DATE
February 26, 2013

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ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
wjbc@wjgnet.com
doi:10.4331/wjbc.v4.i1.1

World J Biol Chem 2013 February 26; 4(1): 1-12
ISSN 1949-8454 (online)

REVIEW

S100B protein in tissue development, repair and regeneration

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Supported by Ministero dell'Università e della Ricerca, No. PRIN 2007LNKSYS, No. 2007AWZTHH_004 and No. 2009WBFZYM_002; Association Française contre les Myopathies, No. Project 12992; Associazione Italiana per la Ricerca sul Cancro, No. Project 6021; and Fondazione Cassa di Risparmio di Perugia, No. 2007.0218.020, No. 2009.020.0021 and No. 2012.0241.021
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Received: December 5, 2012 Revised: January 26, 2013
Accepted: February 25, 2013
Published online: February 26, 2013

Abstract

The Ca^{2+}-binding protein of the EF-hand type, S100B, exerts both intracellular and extracellular regulatory activities. As an intracellular regulator, S100B is involved in the regulation of energy metabolism, transcription, protein phosphorylation, cell proliferation, survival, differentiation and motility, and Ca^{2+} homeostasis, by interacting with a wide array of proteins (*i.e.*, enzymes, enzyme substrates, cytoskeletal subunits, scaffold/adaptor proteins, transcription factors, ubiquitin E3 ligases, ion channels) in a restricted number of cell types. As an extracellular signal, S100B engages the pattern recognition receptor, receptor for advanced glycation end-products (RAGE), on immune cells as well as on neuronal, astrocytic and microglial cells, vascular smooth muscle cells, skeletal myoblasts and cardiomyocytes. However, RAGE may not be the sole receptor activated by S100B, the protein being able to enhance bFGF-FGFR1 signaling by interacting with FGFR1-bound bFGF in particular cell types. Moreover, extracellular effects of S100B vary depending on its local concentration. Increasing evidence suggests that at the concentration found in extracellular fluids in normal physiological conditions and locally upon acute tissue injury, which is up to a few nM levels, S100B exerts trophic effects in the central and peripheral nervous system and in skeletal muscle tissue thus participating in tissue homeostasis. The present commentary summarizes results implicating intracellular and extracellular S100B in tissue development, repair and regeneration.

Key words: S100B; Cell proliferation; Cell differentiation; Cell survival; Cell motility; Development; Tissue homeostasis; Tissue repair; Tissue regeneration

Sorci G, Riuzzi F, Arcuri C, Tubaro C, Bianchi R, Giambanco I, Donato R. S100B protein in tissue development, repair and regeneration. *World J Biol Chem* 2013; 4(1): 1-12 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i1/1.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i1.1

INTRODUCTION

S100B belongs to a multigenic family of small (mol. wt. between 9 kDa and 14 kDa) Ca^{2+}-binding proteins of the EF-hand type comprising more than 20 members exclusively expressed in vertebrates[1,2]. Like other members of this protein family, S100B is expressed in a cell-specific manner; astrocytes, oligodendrocytes, neural progenitor cells, certain neuronal populations, ependymocytes, Schwann cells, enteric glial cells, melanocytes, kidney epithelial cells, adipocytes, chondrocytes, skin Langerhans cells, a subpopulation of lymphocytes, muscle satellite cells, skeletal myofibers, pituitary folliculo-stellate cells and Leydig cells in the testis are the cell types with the

highest expression of S100B. However, at least cardiomyocytes, which normally do not express the protein, do express S100B post-infarction, and in several cell types S100B expression is upregulated in pathological conditions.

Within cells S100B exists in the form of a homodimer, sometime as an S100B-S100A1 heterodimer, in which the two subunits are arranged in an antiparallel fashion[1,3]. Like the majority of S100 members, S100B is a Ca^{2+} sensor protein that becomes activated by Ca^{2+} on the occasion of Ca^{2+} transients. Ca^{2+} induces a relatively large conformational changes in S100B C-terminal half resulting in the exposure of a hydrophobic patch through which the protein interacts with a wide array of target proteins (*e.g.*, enzymes, enzyme substrates, cytoskeletal proteins, adaptor/scaffold proteins, transcription factors, ion channels and ubiquitin E3 ligases) thereby regulating their activities. Thus, S100B is involved in the regulation of energy metabolism, transcription, protein phosphorylation, cell proliferation, survival, differentiation and locomotion, and Ca^{2+} homeostasis.

However, S100B can also exert extracellular effects being secreted by certain cell types (*e.g.*, astrocytes and adipocytes) or passively released by several cell types upon tissue injury. In this latter context S100B can be viewed as a damage-associated molecular pattern (DAMP) or alarmin, *i.e.*, a danger signal capable of activating cells of the innate immune system[1,3-6]. Extracellular effects of S100B mostly have been studied in the context of the central nervous system likely because of its high abundance in the brain and the identification of neurons, astrocytes and microglia as its target cells. Indeed, extracellular S100B has long been implicated in the pathophysiology of Alzheimer's disease and neuroinflammation largely via engagement of the receptor for advanced glycation endproducts (RAGE). However, accumulating evidence suggests that effects of extracellular S100B are not restricted to the brain or to cells of the innate immune system, and that RAGE may not be the sole receptor transducing S100B effects. In the present commentary we shall discuss results implicating intracellular and extracellular S100B in tissue development, homeostasis, repair and regeneration.

S100B IN CELL PROLIFERATION AND DIFFERENTIATION

S100B is involved in cell proliferation, survival and differentiation both as an intracellular regulator and an extracellular signal. Within cells S100B binds to and activates Ndr (nuclear Dbf2-related)[7], a serine/threonine protein kinase implicated in the regulation of cell division and morphology[8]. Regulation of Ndr by S100B involves a conformational change in the catalytic domain triggered by Ca^{2+}/S100B binding to the junction region[9]. However, although S100B-dependent activation of Ndr in cell lines has been documented[7] and in non-dividing and dividing cells S100B localizes to centrosomes[10], which are Ndr targets[8], no evidence has been presented that S100B-dependent activation of Ndr results in stimulation of cell proliferation and/or changes in cell morphology.

S100B also interacts with the tumor suppressor, p53, inhibiting its phosphorylation and tetramerization , *i.e.*, its activation[11-14]. Also, S100B reduces p53 levels[15], and in turn, p53 upregulates S100B expression in melanoma cells[15]. In this scenario, p53 would reduce its own abundance by upregulating its inhibitor, S100B, which would result in uncontrolled proliferation[15] and reduced apoptosis[16] at least in melanoma cells (Figure 1A). However, phosphorylation of specific serine and/or threonine residues in p53 reduces the affinity of the S100B-p53 interaction by an order of magnitude, and is important for protecting p53 from S100B-dependent downregulation[17]. Thus, the S100B overall effect on p53 is likely to reflect a balance between inhibitory cues and intervening biochemical events (*e.g.*, p53 phosphorylation). However, conflicting conclusions have been reported regarding functional implications of S100B/p53 interactions[15,18,19], and it is not known whether these interactions are relevant for tumor progression in other cancers and in non-neoplastic cells. In addition, it has been suggested that by interacting with the ubiquitin E3 ligases, MDM2 (HDM2) and MDM4 (HDM4)[17,20], that are central negative regulators of p53[21], S100B may actually promote p53 activities[20], which adds another layer of complexity to S100B-p53 interactions (Figure 1A). We have shown that forced expression of S100B in neuronal PC12 cells has no effects on p53 levels or nuclear translocation, and it results in enhanced proliferation and reduced differentiation and oxidative stress-induced apoptosis via activation of a PI3K/Akt/$p21^{WAF1}$/cyclin D1/cdk4/Rb/E2F pathway in the absence of serum mitogens[22] (Figure 1B). S100B-dependent reduction of stress-induced apoptosis may also occur via interaction with and activation of the tetratricopeptide repeat protein, PP5, a member of the PPP family of serine/threonine phosphatases[23].

S100B is expressed in proliferating myoblast cell lines[24] and quiescent muscle satellite cells[25], the most relevant stem cell population in adult skeletal muscle tissue[26]. Increasing S100B levels in myoblast cell lines results in no effects on the proliferation rate of asynchronously proliferating myoblasts; however, S100B-overexpressing myoblasts are more resistant to basal and H_2O_2-induced apoptosis in an IκB kinase β (IKKβ)/nuclear factor κB (NF-κB)-mediated manner[27] (Figure 1C). Thus, increasing S100B levels in myoblasts results in augmented cell numbers in consequence of their increased survival rate in stress conditions. Moreover, S100B-overexpressing myoblasts are less prone to acquire mitotic quiescence and proliferate faster than control cells upon re-exposure to serum mitogens after quiescence[27]. Proliferation of muscle satellite cells and their resistance to death-inducing stimuli are critical for efficient muscle regeneration as well as for successful cell therapy of muscular dystrophy[26,28-30]. Thus, intracellular S100B may contribute to muscle regeneration by reducing apoptosis

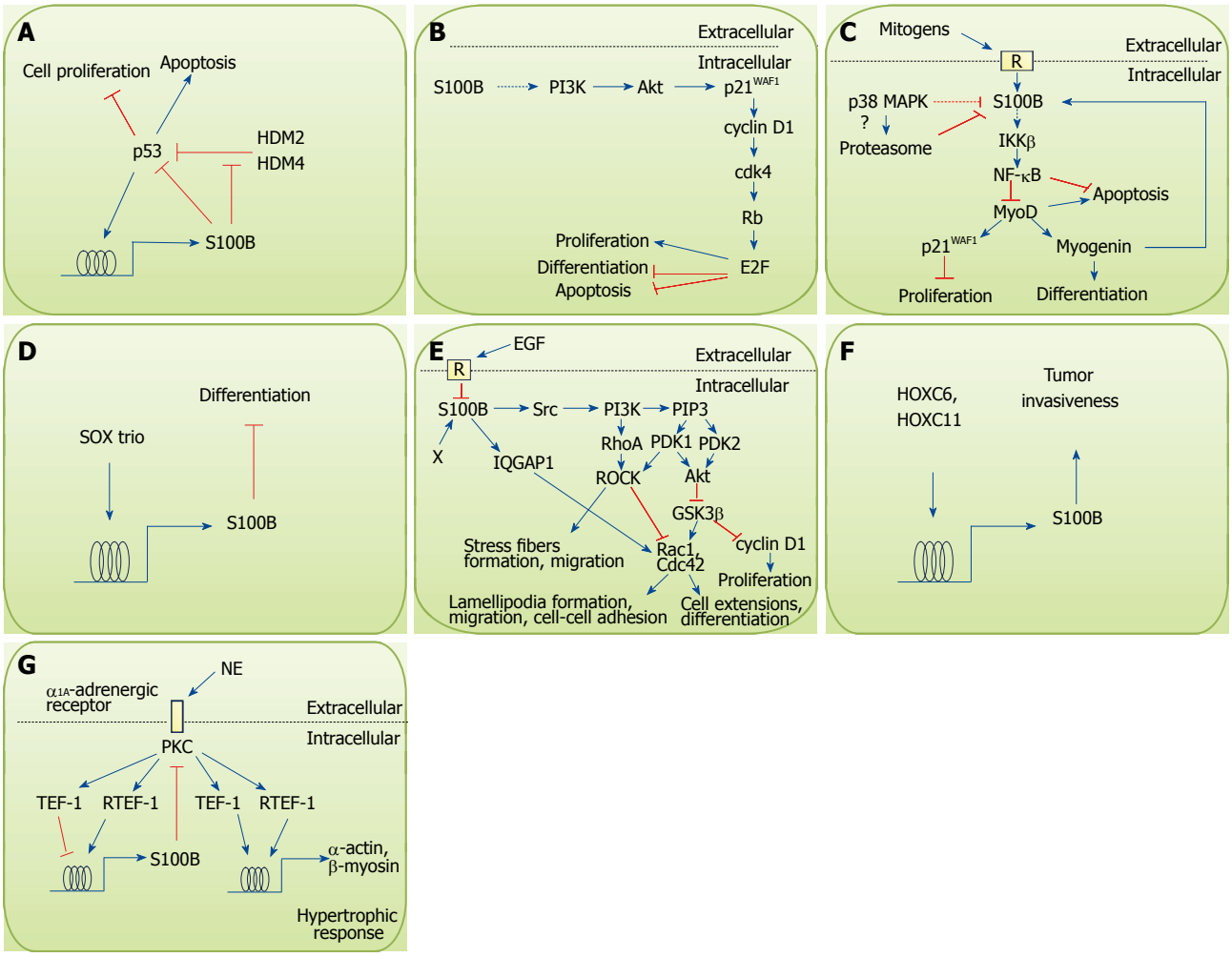

Figure 1 Effects of intracellular S100B on cell proliferation, differentiation, survival and motility. A: Schematics of S100B-p53 interactions in melanoma cells. p53 induces S100B that in turn blunts p53 inhibitory effects on proliferation and stimulatory effects on apoptosis. However, S100B interaction with the ubiquitin E3 ligases, HMD2 and HMD4, may inhibit HMD2/HMD4-dependent reduction of p53 levels; B: Expression of S100B in PC12 neuronal cells results in stimulation of proliferation and inhibition of NGF-induced differentiation and oxidative stress-induced apoptosis *via* activation of the PI3K/Akt pathway; C: Intracellular S100B protects myoblasts against oxidative stress-induced apoptosis and inhibits differentiation *via* activation of the IκB kinase β/ nuclear factor κB (NF-κB) pathway. Also, early after the transfer of myoblasts from growth medium to differentiation medium S100B becomes downregulated by the decrease in serum mitogens and activation of the promyogenic p38 MAPK, that likely stimulates S100B proteasomal degradation. However, S100B becomes re-expressed in differentiated myoblasts under the action of myogenin; D: S100B is induced in chondroblasts by the SOX trio and inhibits differentiation; E: S100B, induced in astrocytic progenitors by an unidentified mechanism (X), interacts with and activates a Src/PI3K pathway that stimulates RhoA/ROCK thereby promoting stress fiber formation and cell migration and Akt thereby inhibiting GSK3β resulting in stimulation of proliferation and inhibition of differentiation. Interaction of S100B with IQGAP1 results in activation of Rac1 responsible for lamellipodia formation during migration. The S100B/IQGAP1/Rac1 interaction may also results in an enhancement of cell-cell adhesion as observed in neurospheres (see text). EGF represses S100B expression during early phases of astrocyte differentiation, which appears to be permissive for astrocytic terminal differentiation. Whether such a mechanism also is operating in cerebellar granule cell progenitors remains to be determined; F: S100B, induced in breast cancer cells by HOXC6 and HOXC11, contributes to tumor invasiveness; G: S100B is induced in post-infarction cardiomyocytes by the norepinephrine/α1A-adrenergic receptor/PKC axis and inhibits hypertrophic response *via* inhibition of PKC

and stimulating the expansion of activated satellite cells (Figure 1C). However, excess expression of S100B in activated satellite cells may be detrimental because its mitogenic effect might interfere with the reconstitution of the satellite cell reserve pool which normally occurs during muscle regeneration and requires that a fraction of cells stop proliferating and enter a quiescent state[26,28,29], and because myoblast proliferation and differentiation are mutually exclusive[26]. Considering that S100B is expressed in high abundance in several cancers[2,3], enhanced expression of S100B in activated muscle satellite cells, from which embryonal rhabdomyosarcomas are thought to originate[31], may also contribute to rhabdomyosarcomagenesis. Preliminary results show that embryonal rhabdomyosarcoma cells do indeed express elevated S100B levels (Riuzzi F, Sorci G, and Donato R, unpublished results).

Collectively, these data suggest that intracellular S100B may intervene in the regulation of proliferation, survival and apoptosis by mechanisms that vary depending on the cell type, the context and, probably, the cell's normal or neoplastic condition. Further work is required to definitely establish the role of S100B in cell proliferation and survival in normal and neoplastic cells and the

molecular mechanism(s) behind S100B overexpression in many cancers.

Intracellular S100B also functions as an inhibitor of differentiation. As mentioned above, expression of S100B in PC12 neuronal cells results in impaired NGF-induced differentiation via activation of a PI3K/Akt/$p21^{WAF1}$/cyclin D1/cdk4/Rb/E2F pathway[22] (Figure 1B). However, induction of S100B expression in NGF-differentiated PC12 neuronal cells does not reverse the differentiated phenotype[22]. Also, S100B is induced in early-stage chondroblast differentiation by the SOX trio and negatively regulates chondrocyte terminal differentiation via an as yet undetermined mechanism[32] (Figure 1D). Interestingly, S100B expression in astrocytic cells is developmentally regulated albeit with different characteristics depending on whether subventricular or cortical astrocytic cells are considered[33]. These studies[33] have established that during the time interval between post-natal days 2 and 8 ramified, differentiating (*i.e.*, GFAP filament-positive) astrocytes are S100B-negative. This suggests that during that time interval S100B may be downregulated and that the protein becomes re-expressed during the final phase(s) of astrocytic differentiation. S100B is expressed in radial glial precursors[34], in the ventricular zone of embryonic mouse cerebellum and progenitors of cerebellar granule cells[35], the protein being expressed in these latter cells as long as they are migrating. S100B interacts with the small GTPase Rac1 and Cdc42 effector, IQGAP1, at the polarized leading edge and areas of membrane ruffling in astrocytoma cell lines[36]. Hence, S100B has been proposed to regulate IQGAP1 activity in relation to cell migration (Figure 1E). In accordance with this view, reduction of S100B levels in astrocyte cell lines and primary astrocytes results in decreased proliferation and migration and acquisition of a differentiated phenotype (*i.e.*, stellation) consequent to reduced activity of a Src/PI3K/RhoA/ROCK pathway and increased activity of the GSK3β/Rac1 module[37] (Figure 1E). These results are consistent with the possibility that repression of S100B expression at certain phases of development of astrocytes and certain neuronal populations may be functionally linked to their differentiation. Thus, S100B may contribute to expand the population of progenitors of neural cells and confer migratory capacity on undifferentiated astrocytes and neuroblasts, and S100B expression has to be repressed for differentiation to take place. In this context, S100B may act to avoid premature differentiation besides promoting cell migration; however, deregulated S100B expression may contribute to gliomagenesis. Intriguingly, knockdown of S100B in the Müller cell line, MIO-M1, results in remarkably inhibited neurosphere formation and differentiation of these cells towards the astrocyte phenotype[37]. Because MIO-M1 neurospheres have been shown to be made of neural precursor cells differentiating towards a neuronal phenotype when cultivated in the presence of bFGF or retinoic acid[38], the results in[37] suggest that S100B may contribute to confer stem cell-like properties on MIO-M1 cells and to reduce their propensity to differentiate into astrocytes. The expression of S100B in the murine cerebellar ventricular zone including the embryonic cerebellar rhombic lip and in cells lining cerebral ventricles[33-35] adds to the possibility that intracellular S100B may contribute to confer pluripotency on precursors of neural cells. Incidentally, the studies in[36,37] highlight S100B's ability to regulate F-actin-based cytoskeleton in an indirect manner, *i.e.*, *via* stimulation of a Src/PI3K/RhoA/ROCK and an IQGAP1/Rac1 pathway, and reduction of the activity of the GSK3β/Rac1 module (Figure 1E), as opposed to the protein's direct effects on microtubule- and intermediate filament-based cytoskeleton[39-43].

On the other hand, HOXC6 and HOXC11, members of homeobox genes that encode transcription factors driving morphogenesis and cell differentiation during embryogenesis[44,45], have been reported to increase transcription of *s100b* in neuroblastoma cells[46] and this was interpreted as indicative of HOXC6 and HOXC11 stimulating differentiation of neuroblastoma cells into Schwann cells through the transcriptional activation of *s100b*. However, in the absence of data on the expression of additional markers such as myelin basic protein or GFAP, the expression of *s100b* may not be itself a proof of cell differentiation towards Schwann cells, oligodendrocytes or astrocytes[22,35,37]. Also, interactions of HOXC11 with the steroid receptor coactivator protein SRC-1, which is a strong predictor of reduced disease-free survival in breast cancer patients, induce the expression of S100B in resistant breast cancer cells[47] (Figure 1F). This latter study supports the notion that expression of S100B in proliferating and/or tumor cells may interfere with differentiation and/or is mechanistically linked to tumor progression. This study[47] also highlights the fact that S100B can be induced in precursors of certain cell types (breast cells, in the present case) and becomes repressed at completion of differentiation; differentiated breast cells do not express the protein whereas persistence of S100B in breast cell precursors may concur to tumor progression and invasion.

S100B is induced in post-infarction cardiomyocytes under the action of norepinephrine and phenylephrine *via* protein kinase C activation thereby limiting the hypertrophic response through the inhibition of the expression of the fetal proteins, skeletal α-actin and β-myosin heavy chain[48-50] (Figure 1G). Accordingly, norepinephrine-induced cardiac hypertrophy is inhibited in S100B transgenic mice[51]. Thus, S100B, which is not expressed in cardiomyocytes in normal physiological conditions, participates in the regulation of cardiomyocyte remodeling after infarction. These results appear in line with the notion that S100B is expressed in cells exhibiting properties of immature cells (post-infarction cardiomyocytes, in the present case). However, similarly to the majority of neuronal cells, in which S100B becomes stably repressed before differentiation, and differently from astrocytes (see above) and myoblasts (see below), in which a transient downregulation of S100B occurs at the beginning of dif-

ferentiation, full maturation of cardiomyocytes is accompanied by stable repression of S100B expression.

Intracellular S100B modulates the differentiation of myoblasts, the precursors of skeletal myofibers. Indeed, overexpression of S100B in myoblasts blocks myogenic differentiation *via* IKKβ/NF-κB-mediated inhibition of expression of the muscle-specific transcription factor, MyoD, and the MyoD-downstream effectors myogenin and $p21^{WAF1}$, and conversely, reduction of S100B expression in myoblasts by siRNA techniques results in reduced NF-κB activity and enhanced myogenic differentiation[25] (Figure 1C). It is known that NF-κB is a negative regulator of myogenic differentiation *via* inhibition of expression and/or reduction of stability of MyoD[52-54]. Also, S100B binds to, and inhibits EAG1 potassium channels Ca^{2+}-dependently[55]. Because these channels have been reported to play a role in myoblast fusion into myotubes[56] it is possible that S100B may negatively affect myoblast differentiation *via* inhibition of EAG1 potassium channels as well. Moreover, compared with young subjects, muscle satellite cells from aged human subjects, which are known to be proliferation and differentiation defective[29,57], express higher levels of S100B and knockdown of S100B in aged satellite cells rescues their myogenic potential in part[58]. Notably, despite their high S100B levels, aged muscle satellite cells show a low proliferation rate and a remarkably reduced ability to secrete S100B and bFGF[58]. However, treatment of aged satellite cells with S100B or bFGF rescues their proliferative potential in part[58]. These results suggest that physiological levels of S100B in activated satellite cells and the satellite cells' ability to secrete the protein concur to optimize the expansion of activated satellite cells required for satellite cell homeostasis, the maintenance of optimal muscular mass and/or efficient skeletal muscle regeneration after acute injury. In this context it is noteworthy that aged human satellite cells also exhibit altered expression of RAGE[58] shown to exert promyogenic effects[59-61] and to be required for S100B secretion[62]. Because transient transfection of aged satellite cells with full-length RAGE rescues their myogenin potential in part[58], one may speculate that the combination of enhanced S100B expression and expression of an altered form of RAGE may contribute significantly to their reduced myogenic potential, hence to sarcopenia. The recent demonstration that levels of bFGF are high and are responsible for disrupted satellite cell quiescence in aged skeletal muscle in homeostatic conditions[63] lend support to the possibility that excess S100B in aged satellite cells, potentially caused by high bFGF[1,3] may ultimately lead to defective muscle regenerative capacity as observed in sarcopenia. Interestingly, levels of S100B decrease in non-fused myoblasts early after their transfer to differentiation medium and S100B becomes re-expressed in differentiating (*i.e.*, myogenin-positive) myocytes[25,27], which supports the notion that S100B levels have to decrease transiently in certain cell types for they to differentiate. Both differentiation cues (namely the activation of the promyogenic p38 MAPK) and reduction of mitogens appear to determine the transient downregulation of S100B in myoblasts in differentiation medium *via* transcriptional and post-translational (proteasome-dependent) mechanisms[27] (Figure 1C). Collectively, these results suggest that S100B in myoblasts contributes to reduce their premature differentiation which would be detrimental to skeletal muscle regeneration after acute injury, and that levels of S100B should be kept within a certain range of abundance in order to avoid excessive expansion of activated satellite cells leading to defective reconstitution of the damaged tissue and the pool of quiescent satellite cells.

Whereas EGF has been reported to reduce S100B expression in developing astrocytes[33] (Figure 1E), the extracellular stimuli and intracellular mechanisms causing transient or stable downregulation of S100B expression during cell differentiation are not completely defined. Also, because mature astrocytes, chondrocytes, myocytes (*i.e.*, differentiated myoblasts), skeletal myofibers and certain neuronal populations in the adult brain express S100B[3,25,64], mechanisms should exist that cause re-expression of the protein at later developmental stages without determining cell de-differentiation[22,27]. In the case of skeletal muscle cells, the muscle-specific transcription factor, myogenin, that is essential for myogenic differentiation[26], has been implicated in the re-expression of S100B in myocytes[27] (Figure 1C). Overall, these observations suggest that functions of S100B may be different in developing and mature cells and that S100B may regulate different signaling pathways and functions depending on the cell type and the cell's status. Future work should dissect the molecular mechanism(s) responsible for the regulation of S100B expression in immature (proliferating) and fully differentiated cells.

Extracellular S100B also regulates cell proliferation, survival and differentiation. Several factors/conditions regulate either positively or negatively S100B secretion by astrocytes, among which are interleukin-1β, extracellular levels of Ca^{2+} and K^{+}, inhibitors of gap junctions, antioxidants, lipopolysaccharide, apomorphine and certain antipsychotic drugs[65-69]. At the low nM concentration found in the brain extracellular space in normal physiological conditions[3], S100B exerts pro-survival effects on neurons[70-73], stimulate astrocyte proliferation[74] and reduce microglial reactivity[75,76], *via* RAGE engagement in most cases (Figure 2). However, at low nM levels S100B synergizes with proinflammatory cytokines to activate microglia[77] suggesting that S100B may switch from antiinflammatory to proinflammatory at early phases of neuroinflammation (*i.e.*, in the presence of low levels of inflammatory cytokines). Yet, attenuation of microglia activity by low concentrations of S100B may contribute to local tumor immunosuppression[76].

S100B has been implicated in the activity of antidepressants. The selective serotonin reuptake inhibitor, fluoxetine, increases S100B content in the hippocampus[78] and stimulates S100B secretion from astrocytes[79] and serotoninergic neurons[80]. It has been shown that

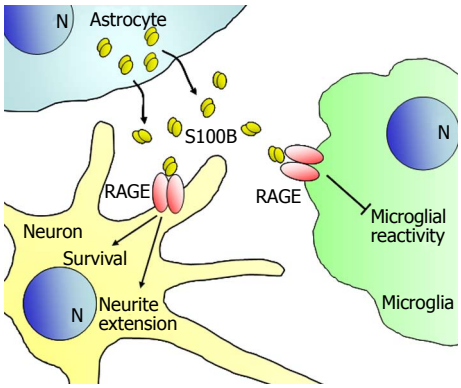

Figure 2 Schematics of effects of low concentrations of S100B on neuronal and microglial cells. By engaging receptor for advanced glycation end-products, S100B exerts trophic effects on neurons and reduces microglia reactivity. RAGE: Receptor for advanced glycation end-products.

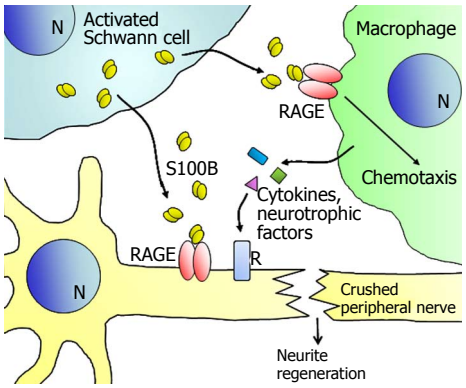

Figure 3 Schematics of effects of low concentrations of S100B on axonal regeneration of crushed peripheral nerves. S100B secreted/released from activated Schwann cells stimulates recruitment of Schwann cells and macrophages to the injury site and release of cytokine and trophic factors leading to axonal regeneration, in a receptor for advanced glycation end-products -dependent manner. RAGE: Receptor for advanced glycation end-products.

secreted S100B downregulates microRNA-16 in noradrenergic neurons, which consequently acquire properties of serotoninergic neurons[80]. Although no information is available regarding the mechanism whereby fluoxetine induces serotoninergic neurons to express and secrete S100B, the mechanism whereby secreted S100B reduces microRNA-16 levels in noradrenergic neurons or the S100B-serotoninergic neuron relationships in S100B-null or transgenic mice, these results point to an important role of extracellular S100B in fluoxetine-dependent neurogenesis and neuronal plasticity[81,82].

Serum levels of S100B increase remarkably following an intense physical exercise[83,84], the source of the protein reasonably being skeletal myofibers in these circumstances. Indeed, intense physical exercise is associated with reversible skeletal muscle tissue damage and release of intracellular proteins[26], and the local concentration of S100B may be even higher than in serum thus allowing paracrine S100B effects on activated muscle stem (satellite) cells. In fact, at picomolar to low nanomolar concentrations S100B inhibits myoblast differentiation and stimulates myoblast proliferation[85-87] raising the possibility that the protein may participate in the process of skeletal muscle regeneration by expanding the myoblast population (see below).

S100B IN TISSUE REGENERATION

Since the discovery that a protein factor purified from brain and endowed with neurite extension activity was a disulfide cross-linked form of S100B[88] and the demonstration that S100B is found in the brain extracellular space[89] and is actively secreted by astrocytes[90], a mess of information has been provided over time on the protective and trophic role of S100B on neurons[70-73,91-98](Figure 2). S100B is found expressed in Schwann cells in uninjured peripheral nerves as well as in activated Schwann cells during the degeneration period of crushed nerves, *i.e.*, up to day 7 post-injury, and in normal Schwann cells reappearing during the regeneration period, *i.e.*, after day 7 post-injury, in the zone of the crush and proximal and distal to it[99]. In similar conditions, RAGE becomes expressed in axons and in infiltrating mononuclear phagocytes and reduction of RAGE expression and/or activity results in suppression of anatomical regeneration and functional recovery[100,101]. Upon acute peripheral nerve injury, S100B released from Schwann cells in damaged nerves activates RAGE in infiltrating macrophages[100,101] and in activated Schwann cells[102]; infiltrating macrophages exert beneficial effects by clearing cell debris and dead neutrophils and releasing cytokines and trophic factors, whereas activated Schwann cells release cytokines and neurotrophic factors shown to be crucial for the repair of injured nerves (Figure 3). S100B-activated RAGE promotes Schwann cell migration during the course of repair of injured peripheral nerves through the induction of thioredoxin interacting protein and activation of p38 MAPK, CREB and NF-κB[102]. S100B also stimulates proliferation and differentiation of neural progenitor cells from the subventricular zone of the adult mouse brain *via* RAGE activation[103]. These results complement the long-standing notion that S100B stimulates neuronal cell survival and differentiation *via* RAGE engagement[71,72].

S100B also is expressed in skeletal myofibers[25,64] from which it is massively released early upon acute injury with declining release during the regeneration phase[104] (Figure 4A). Released S100B stimulates myoblast proliferation and concomitantly activates the myogenic differentiation program *via* RAGE engagement early after injury (Figure 4B), *i.e.*, at a time when myoblast density and the level of released bFGF are low[104], thereby contributing to the timely and limited expansion of the myoblast population required for efficient muscle regeneration. Indeed, acutely injured *Rage*$^{-/-}$ muscles show delayed regeneration[61]. However, persistence of extracellular S100B in the damaged tissue is likely to prolong the myoblast proliferation phase at the expense of differentiation and reconstitution of the pool of quiescent satellite cells *via* enhancement of bFGF/FGFR1 signaling and blockade of RAGE

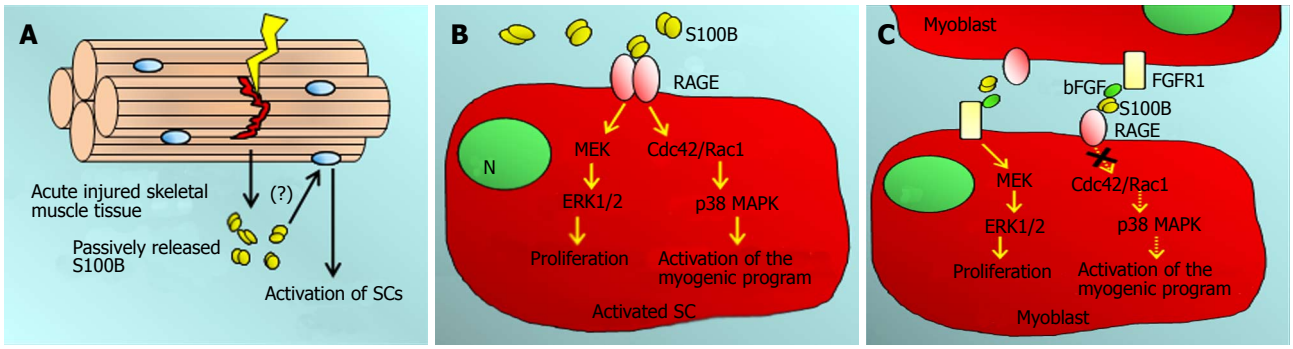

Figure 4 Effects of S100B in skeletal muscle regeneration. A: S100B is passively released from acutely injured skeletal muscle tissue early after injury. Whether S100B activates quiescent muscle satellite cells (SCs) is not known (?); B: Released S100B may stimulate myoblast proliferation and simultaneously activate the myogenic program *via* receptor for advanced glycation end-products (RAGE) engagement, during the next few days post-injury (early regeneration phase); C: However, during the intermediate regeneration phase (*i.e.*, from day 3 to day 7 post-injury, in coincidence with the peak of released bFGF and the myoblast proliferation phase), S100B may enhance bFGF-FGFR1 mitogenic signaling thereby contributing to expand the myoblast population while simultaneously inactivating its canonical receptor, RAGE. RAGE: Receptor for advanced glycation end-products.

signaling[87,104] (Figure 4C). The switch of S100B from a RAGE-activating factor to a bFGF/FGFR1 activating factor depends on the S100B concentration, the presence of bFGF and myoblast density[87,104]. Current findings indicate that neutralization of released S100B in acutely injured wild-type skeletal muscles results in defective regeneration as a consequence of reduced expansion of the population of activated satellite cells, reduced infiltration of the injured tissue with macrophages and delayed transition of macrophages from the M1 (proinflammatory) to the M2 (antiinflammatory) phase (Riuzzi F, Sorci G, Beccafico S and Donato R, in preparation). These results indicate that released S100B participates in the regeneration of acutely injured muscles by stimulating myoblast proliferation, macrophage infiltration and macrophage transition from a proinflammatory phenotype to an antiinflammatory phenotype. Our ongoing results also show that these effects of S100B are strictly RAGE-dependent, because neutralization of released S100B in acutely injured *Rage*$^{-/-}$ muscles does not change the muscle regeneration pattern described in[61]. However, one may anticipate that chronic release of S100B from skeletal myofibers in, *e.g.*, muscular dystrophies and chronic inflammatory muscle diseases may translate into high local S100B concentrations amplifying or perpetuating muscle damage, a situation reminiscent of what occurs in the brain where low S100B levels are beneficial whereas chronically high S100B levels are detrimental, *via* RAGE engagement in both cases[1,3,71,72].

Also, cell/tissue identity appears to profoundly condition S100B's extracellular effects. For example, whereas at concentrations $\leqslant$ 50 nmol/L S100B exerts trophic effects on neuronal and astrocytic cells and skeletal myoblasts[3,105,106], at doses $\geqslant$ 50 nmol/L the protein causes RAGE-dependent cardiomyocyte apoptosis[107]. However, a short-term (1 h) treatment of cardiomyocytes with S100B (100 nmol/L) (a condition insufficient to cause apoptosis) results in a RAGE-dependent secretion of vascular endothelial growth factor (VEGF) which in turn induces myofibroblast proliferation[108]. By this mechanism S100B might contribute to post-infarction scar formation, a kind of tissue reparative process. Whether S100B also causes VEGF-dependent post-infarction neoangiogenesis remains to be investigated. Intriguingly, whereas S100B is induced in the heart of diabetic mice as well, S100B mRNA and protein expression levels decrease in diabetes post-infarction by a mechanism that remains to be identified, and deletion of *s100b* has a deleterious effect on cardiac function in this condition partly attributed to increased ventricular dilation associated with increased AGE formation and reduced GLUT4 expression, *i.e.*, reduced cardiac glucose metabolism[109]. Whether these changes are due to reduced intracellular or extracellular effects of S100B is not known. Yet, these results point to a protective role of S100B in post-infarction heart.

S100B IN RESOLUTION OF INFLAMMATION

The role of extracellular S100B as a DAMP involved in inflammation is an accepted notion (see Refs.[1,3-6,106] for pertinent literature). However, for S100B to sustain inflammation *via* activation of macrophages/microglia it has to be present at relatively high concentration at injury sites[1,3-6,106,110], as it reasonably occurs during the course of chronic tissue damage as a result of a continuous release of the protein from injured cells, cell necrosis and/or defective clearance. However, recent evidence points to a novel role of S100B in resolution of inflammation in *Aspergillus fumigatus* infection in lung[111]. TLR2 activation on bronchial epithelial cells by the fungus results in upregulation of expression and release of S100B, that paracrinally binds to RAGE on polymorphonuclear neutrophils and mediates its association with TLR2 for subsequent inhibition. In addition, S100B upon binding to nucleic acids in bronchial epithelial cells, also activates an intracellular TLR3/TLR9/TRIF-dependent pathway leading to repression of *s100b* transcription. The transcriptional repression of *s100b* by the sequential action of down-

stream MyD88- and TRIF-dependent NF-κB signaling pathways[111] thus provides the molecular basis for a braking circuit in infection whereby the endogenous danger protects the host against pathogen-induced inflammation and a nucleic acid-sensing mechanism resolves danger-induced chronic inflammation. Whether this is a general mechanism of action of the S100B/RAGE axis in the course of infections remains to be determined. However, high local S100B concentrations exacerbates *Aspergillus fumigatus*-induced pulmonary inflammation[111] likely *via* sustained stimulation of RAGE signaling. Interestingly, the *S100B* (+427C/T) polymorphism results in S100B overexpression which associates with susceptibility to invasive aspergillosis in patients undergoing hematopoietic stem cell transplantation whenever the recipients show *RAGE* (-374T/A) polymorphism resulting in RAGE overexpression[112].

CONCLUDING REMARKS

During the last decade there has been a burst of interest in S100B functions[3,6,106] following the seminal demonstration that S100B engages RAGE in immune cells and behaves like a DAMP[113]. Evidence has been provided shortly after that both the neurotrophic and neurotoxic effects of low and high S100B levels, respectively, on neuronal cells[1,3], are mediated by RAGE engagement[71]. However, S100B mostly has been viewed as a DAMP involved in the inflammatory response and S100B often has been used as a generic RAGE activator in the context of the inflammatory response[3,6,113].

Yet, a large body of information indicates that S100B protein is involved in cell proliferation, survival, motility and differentiation by acting as an intracellular regulator and an extracellular signal in normal physiological conditions and during the acute phase of tissue damage. In so doing, S100B may play a role in tissue development and repair after acute injury, through the refinement or fine tuning of enzyme activities, the dynamics of the cytoskeleton and cell-specific gene expression, and responses to external stimuli. Moreover, S100B exerts anti-infection effects in the bronchial epithelium where a tight regulation of its expression and release is mechanistically linked to the resolution of inflammation after fungal infection. Future work should assess the molecular mechanism(s) regulating S100B expression in developing and mature cells and during tissue repair/regeneration.

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World Journal of Biological Chemistry

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ISSN 1949-8454 (online)

Launch date

July 26, 2010

Frequency

Quarterly

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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2013 May 26; 4(2): 13-34

Baishideng Publishing Group
www.wjgnet.com

WJBC

World Journal of Biological Chemistry

Contents

Quarterly Volume 4 Number 2 May 26, 2013

Contents

World Journal of Biological Chemistry
Volume 4 Number 2 May 26, 2013

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World Journal of Biological Chemistry is now indexed in PubMed Central, PubMed, Digital Object Identifier, and Directory of Open Access Journals.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Quarterly

EDITOR-IN-CHIEF
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World Journal of Biological Chemistry
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No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
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http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road, Wan Chai,
Hong Kong, China
Telephone: +852-6555-7188
Fax: +852-3177-9906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLICATION DATE
May 26, 2013

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1949-8454/g_info_20100316155305.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

WJBC World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
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doi:10.4331/wjbc.v4.i2.13

World J Biol Chem 2013 May 26; 4(2): 13-15
ISSN 1949-8454 (online)

FIELD OF VISION

At the dawn of a new revolution in life sciences

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Received: February 8, 2013 Revised: March 25, 2013
Accepted: April 3, 2013
Published online: May 26, 2013

Abstract

In a recently published article Sydney Brenner argued that the most relevant scientific revolution in biology at his time was the breakthrough of the role of "information" in biology. The fundamental concept that integrates this new biological "information" with matter and energy is the universal Turing machine and von Neumann's self-reproducing machines. In this article we demonstrate that in contrast to Turing/von Neumann machines living cells can really reproduce themselves. Additionally current knowledge on the roles of non-coding RNAs indicates a radical violation of the central dogma of molecular biology and opens the way to a new revolution in life sciences.

Key words: History of science; Paradigm shift; Information; Non-coding RNAs

Core tip: Sydney Brenner describes the radical revolution in life sciences during his lifetime: the occupation of biology by quantum mechanics, concerning the fundamental questions of matter and energy followed by the rise of genetics that showed that chromosomes were the carriers of genes. Biology is, in this respect, physics with computation, *i.e.*, the bottom-top approach in biology is sufficient to solve all our goals in life science. In contrast to this we demonstrate, that biology and life is not only physics and digital information encoded in DNA sequences. In order to understand life in its whole complexity, the top-bottom processes such as occurs in epigenetics and non-coding RNA regulations leads to a new revolution in life sciences.

Baluška F, Witzany G. At the dawn of a new revolution in life sciences. *World J Biol Chem* 2013; 4(2): 13-15 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i2/13.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i2.13

COMMENTARY ON HOT TOPICS

In a history of science perspective, Sydney Brenner reminds us on the revolutions in the life sciences[1]. The remarkable aspect of sciences, as articulated by Kuhn[2], is that the most relevant progress does not occur by small steps but, rather, by revolutionary changes. These so-called paradigm shifts do not reject the former mainstream paradigms but integrate them, as a small part of reality, into the most recent empirical data in a coherent manner.

Kuhn[2] described the scientific revolutions as periodic social patterns resulting from accumulation of anomalies not predicted, and explainable, *via* so-called normal science. The dominating mainstream paradigm is the leading background for spreading and teaching scientific knowledge in school and university curricula. Even if new empirical data does not fit into the realm, mainstream proponents insist in this explanatory model. This remains true even if more and more empirical data does not fit into this realm. Then, new concepts, new insights and revolutionary new ideas are published that could integrate this new available data also. Mainstream proponents become sometimes aggressive and reject new concepts not by stringent arguments but rather by dogmatic insistence. As Brenner[1] mentioned, half a century before Kuhn[2] developed his theoretical but empirically proved theory of

the progress of scientific knowledge, Max Planck pointed out that this pattern of confrontation has not been solved by exchange of good arguments[1]. In contrast to this new paradigms succeed because the proponents of the old one grow old and die, *i.e.*, it is a natural not a rational solution: "A new scientific truth does not triumph by convincing opponents and making them see the light, but rather because its opponents eventually die, and a new generation grows up that is familiar with it"[3]. Then the revolutionary new paradigm becomes the mainstream paradigm and all the teaching curricula become adapted, until new empirical data not compatible with the ruling paradigm, start to repeat this process again.

Brenner describes the radical revolution in life sciences during his lifetime: the occupation of biology by quantum mechanics, concerning the fundamental questions of matter and energy followed by the rise of genetics that showed that chromosomes were the carriers of genes. Brenner calls it the big error of physicist Erwin Schrödinger, who speculated on the physical nature of the genetic material, in that he assumed that "chromosomes not only contained the plan for the development of the organism but also had the means to execute it." The discovery of the double helix resulted in the acceptance of new paradigm that information is physically embodied in DNA sequences of four different bases[1]. In contrast to the time before 1953, the question of information became central. The components of DNA are simple chemicals, but the biological complexity that can be generated by the information of different sequences is revolutionary. The fundamental concept that integrates this new biological "information" with matter and energy is the universal Turing machine and von Neumann's self-reproducing machines[4]. According to Brenner, it was the fundamental error of Erwin Schrödinger that he considered the chromosomes to combine both 'the architect's plan and builder's craft in one'; as the chromosomes do not contain the means for the execution of organismal plan, but only a description of these means[4]. Consequently it follows that biology is, in fact, physics with computation[1,4]. In other words, the bottom-top approach in biology is sufficient to solve all our goals in life science, culminating in the generation of artificial intelligence in future.

But is this really true? The universal Turing machine and the self-reproducing machines of von Neumann still remain at the conceptual stage. However, no single self-reproducing machine had ever been observed within the last 80 years. There are good reasons for this, because it is, in principle, impossible that an artificial machine could reproduce itself[5]. In contrast to the artificial machines which cannot reproduce themselves, the living cells and organisms can reproduce itself and - additionally, generate an abundance of behavioral motifs for which no algorithm can be constructed, such as *de novo* generation of coherent nucleotide sequences[5]. As inherent part of new revolution in life sciences, it emerges that genetic information in living cells is not the result of statistical errors in reproduction of DNA, or random assemblies of nucleotides which are subject to selection. As we know today, an abundance of RNA based agents are evolutionary genetic content operators[6-8]. Moreover, it is RNA, not DNA, which decides about gene expression, both from the temporal as well as spatial perspective[6-8]. Most recent empirical data show convincingly that infectious agents such as viruses, mobile genetic elements and an abundance of non coding RNAs serve as basic tools for generation of genetic novelties, variations and - most important - their regulations[6-9].

Now we know that DNA, which is packaged into the epigenetically marked chromosomes, contains the genetic information as well as the abundance of non-coding sequences, proteins, and RNAs that regulate, also *via* control of chromatin assembly and higher-order structures, gene expression, replication, transcription, translation, repair and epigenetic markings[5-9]. Non-coding DNAs, firstly denoted as junk, are playing central roles in genome organization and evolution[7-9]. In addition, the central dogma of molecular biology[10], according which there is only one way of the biological information transfer (from DNAs, *via* RNAs, to proteins), is refuted recently on basis protein-based analog heredity and non-random adaptive mutations[7-9,11]. Besides the digitally-coded heredity *via* coding DNA sequences, there are several layers of analog inheritance in which proteins, structural templates, and agent-based active organismal behavior feedback in a top-bottom manner back to the genome[5-8]. As epigenetic variation precedes and facilitates genetic adaptation, the analog-based protein-conformation-mediated inheritance is representing the most radical violation[11] of the Central Dogma of molecular biology[10].

One example is the role of noncoding RNAs in neuronal plasticity, the prerequisite of learning and memory-based adaptation in contrast to genetically determined behavior: Non-coding RNAs can be regulated in a varying manner, coordinated or independently, autonomously or functionally interrelated. They can regulate individual genes as well as large genetic networks. They can precisely control spatiotemporal deployment of genes that are executing neuronal processes with extreme cell specifity. Various classes of non-coding RNAs target each other for post-transcriptional regulation *via* alternative splicing, polyadenylation, 5' capping, non-templated modifications and RNA editing. Especially RNA-editing can transmit environmental information to the epigenome and therefore enables neuronal plasticity with learning and memory[12].

The second example is how epigenetic imprinting regulates gene expression. Several classes of macro non-coding RNAs are active in DNA methylation, generally active in clustered genes throughout the genome. Genetic imprinting serves as effective tool in gene silencing and is a crucial regulatory network to tissue specific expression in replication. The whole variety of spatiotemporal coherent expression patterns especially in complex organisms with its variety of tissues depends on these epigenetic regulations. According adaptational purposes such as extreme preda-

tor-pray stress situations, nutrition availability or dramatic change in environmental circumstances (temperature), epigenetic marking may change and therefore represents a top -bottom regulatory network[13-15].

As predicted by Thomas Kuhn in his book, the adherents of the Central Dogma still cling firmly to previous paradigm, even accusing some proponents of the new view of life sciences being linked to the "intelligent design" creationist community. However, it is rather the dogmatic approach of these passing paradigm scientists, which inhibits dynamic advances of sciences, adding fuel to nonscientific worldviews such as that promoted by adherents of the "intelligent design". In fact, dogmatic thinking is not compatible with the curiosity-driven sciences[16].

In conclusion, contemporary biology is accomplishing current revolution in life sciences. It is getting obvious that biology and life is not only physics and digital information encoded in DNA sequences. In order to understand life in its whole complexity, the top-bottom processes and analog information are essential[17]. A new revolution in life sciences[5-9,11] will integrate current empirical data, not fitting into the present mainstream science, into a new conceptual realm which cannot be provided by the Turing/von Neumann machines[5].

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P- Reviewers Passi A, NumakawaT, Müller W
S- Editor Wang JL **L- Editor** A **E- Editor** Lu YJ

WJBC World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
wjbc@wjgnet.com
doi:10.4331/wjbc.v4.i2.16

World J Biol Chem 2013 May 26; 4(2): 16-17
ISSN 1949-8454 (online)

MINIREVIEW

Heart and Brain: A neutro-genomic link

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Received: February 18, 2013 Revised: April 23, 2013
Accepted: April 28, 2013
Published online: May 26, 2013

Abstract

The philosophy of heart and brain are very ancient in our literature where the things good for the heart are not suggested good for the brain and vice-versa. Modern medicine is characterized by a high degree of specialization and the heart-brain connection that could be targeted to treat these complex cardiovascular/brain disorders. The idea that adverse diet/genome interactions can cause disease is not new. In the recent era the science of nutritional genomics have increased our understanding of diet-health-gene interactions and have provided a number of benefits for individuals, groups and societies. Since dietary chemicals are regularly ingested and participate indirectly and directly in regulating gene expression, it follows that a subset of genes regulated by diet must be involved in disease initiation, progression, and severity. In this regards Liver X Receptor (LXR)-α, a key transcription factors, associated with the several chronic pathological situation including coronary heart disease and neurodegenerative diseases have recently been found to be regulated by the dietary components. The crucial findings at molecular biology unit, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, INDIA have not only forced us to explore nutritional genomics as a holistic systems approach to understand the relationship between diet and health, but also to look into the disease preventing and health promoting foods that match our lifestyles, cultures and genetics. After all, we are what we eat.

Key words: Heart; Brain; Nutrition; Gene interaction; Liver X Receptor

Core tip: The progression from a healthy phenotype to a chronic disease phenotype comes into existence by abnormal regulation of gene expression, influenced by the dietary components and gene environment interaction. Liver X Receptor-α is one of the key transcription factor which is modulated by the dietary components such as oxycholesterol, withaferin A, vitamin C, vitamin D and statins. Thus the molecule attracts its role in the field of neutro-genomics.

Dave VP, Kaul D. Heart and Brain: A neutro-genomic link. *World J Biol Chem* 2013; 4(2): 16-17 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i2/16.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i2.16

INTRODUCTION

The philosophy of heart and brain are very ancient in our literature where the things good for the heart are not suggested good for the brain and vice-versa. Modern medicine is characterized by a high degree of specialization and the heart-brain connection that could be targeted to treat these complex cardiovascular/brain disorders[1]. In the recent era the science of nutritional genomics have increased our understanding of diet-health-gene interactions and have provided a number of benefits for individuals, groups and societies[2].

Here, I would like to share my experience at Dr. Kaul's molecular biology unit, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, INDIA, where I have become the witness of some crucial findings which correlates the heart and brain at neutro-genomic level.

LIVER X RECEPTOR-α: NEUTRO-GENOMIC LINKER BETWEEN HEART AND BRAIN

Under the light of the existing literature in the field of cardiovascular as well as neurodegenerative diseases, ligand activated nuclear receptor Liver X Receptor-α (LXR-α) have caught imagination of researcher for its ability to regulate an array of genes involved in lipid metabolism, inflammation, glucose homeostasis and innate immunity[3]. LXR-α is highly expressed in the normolipidemic and hyperlipidemic coronary heart disease subjects which shows a nature's protective role against the disease[4] but due to presence of inherent genetic aberration in such subjects this molecule is not been able to protect the disease[5]. Interestingly reports from Molecular Biology Unit, PGIMER, Chandigarh, INDIA show that vitamin C and statins increases the expression of LXR[6], whereas vitamin D3 can serve as an alternative ligand for the aberrant form of LXR-α and thus can restore its functional abnormality[7]. Further statins which are the best drug of choice to treat cardiovascular patients, also increases the serum vitamin D3 level and thus they serves to increases the expression as well as provides the functional ligands for aberrant LXR-α[7]. The observation also supports to the fact that low level of vitamin D3 is associated with higher risk of coronary heart disease[8]. Withaferin A which is a dietary component isolated from Withania somnifera also act as a ligand for LXR-α[5]. Thus by altering the dietary components, which may facilitate LXR-α activation can lead to regression in the development of cardiovascular diseases.

Withania somnifera is classified in Ayurveda (ancient Hindu system of medicine) as a *rasayana*, a group of plant-derived drugs reputed to promote physical and mental health, augment resistance of the body against disease and diverse environmental factors, revitalizes the body in debilitated conditions and increases longevity[9,10]. Interesting reports from molecular biology unit at PGIMER, Chandigarh, INDIA shows that exposure of neuroblastoma cells with LXR agonist like Withaferin A and 24(S) hydroxycholesterol connects LXR-α activation with the genes recognised to be involved in the regulation of aberrant beta amyloid production leading to the generation of toxic and inflammatory mediators responsible for neuronal death, a hallmark of Alzheimer disease[11]. This fact is correlated by the observation that cerebrospinal fluid of Alzheimer's patient possesses increases 24(S)-hydroxycholesterol compared to healthy controls[12].

CONCLUSION

Thus the findings have not only forced us to explore nutritional genomics as a holistic systems approach to understand the relationship between diet and health, but also to look into the disease preventing and health promoting foods that match our lifestyles, cultures and genetics. After all, we are what we eat.

ACKNOWLEDGEMENTS

I am thankful to Professor Deepak Kaul, Head, Department of Experiment Medicine and Biotechnology, PGIMER, Chandigarh, INDIA for providing me all the facilities for my doctoral studies as well as giving me opportunity to get me exposed with the several crucial findings in the field of Nutritional/Molecular Medicine; Vivek Priy Dave.

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World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
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doi:10.4331/wjbc.v4.i2.18

World J Biol Chem 2013 May 26; 4(2): 18-29
ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Refractoriness of interferon-beta signaling through NOD1 pathway in mouse respiratory epithelial cells using the anticancer xanthone compound

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Received: January 14, 2013 Revised: March 19, 2013
Accepted: April 28, 2013
Published online: May 26, 2013

Abstract

AIM: To explore the possibility that nucleotide oligomerization domain 1 (NOD1) pathway involved in refractoriness of interferon-β signaling in mouse respiratory epithelial cells induced by the anticancer xanthone compound, 5,6-dimethylxanthenone-4-acetic acid (DMXAA).

METHODS: C10 mouse bronchial epithelial cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, 100 g/mL streptomycin. Pathogen-free female BALB/c mice were used to explore the mechanisms of refractoriness of interferon-signaling. Mouse thioglycollate-elicited peritoneal macrophages, bone marrow derived macrophages and bone marrow derived dendritic cells were collected and cultured. The amount of interferon (IFN)-inducible protein-10 (IP10/CXCL10), macrophage chemotactic protein (MCP1/CCL2) and interleukin (IL)-6 secreted by cells activated by DMXAA was quantified using enzyme-linked immunosorbent assay kits according to the instructions of the manufacturers. Total RNA was isolated from cells or nasal epithelium with RNeasy Plus Mini Kit, and cDNA was synthesized. Gene expression was checked using Applied Biosystems StepOne Real-Time Polymerase Chain Reaction System. Transfection of small interfering RNA (siRNA) control, NOD1 duplexed RNA oligonucleotides, and high-mobility group box 1/2/3 (HMGB1/2/3) siRNA was performed using siRNA transfection reagent.

RESULTS: DMXAA activates IFN-β pathway with high level of IFN-β dependent antiviral genes including 2′, 5′-oligoadenylate synthetase 1 and myxovirus resistance 1 in mouse thioglycollate-elicited peritoneal macrophages, bone marrow derived macrophages and bone marrow derived dendritic cells. Activation of IFN-β by DMXAA involved in NOD1, but not HMGB1/2/3 signal pathway demonstrated by siRNA. NOD1 pathway plays an important role in refractoriness of IFN-β signaling induced by DMXAA in mouse C10 respiratory epithelial cells and BALB/c mice nasal epithelia. These data indicate that DMXAA is not well adapted to the intrinsic properties of IFN-β signaling. Approaches to restore sensitivity of IFN-β signaling by find other xanthone compounds may function similarly, could enhance the efficacy of protection from influenza pneumonia and potentially in other respiratory viral infections.

CONCLUSION: NOD1 pathway may play an important role in refractoriness of IFN-β signaling in mouse respiratory epithelial cells induced by DMXAA.

Key words: Innate immunity; Interferon; Refractoriness; Xanthone; Bronchial epithelium

Core tip: We recently demonstrated that a small, cell-

permeable compound, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), was able to induce production of interferon (IFN)-γ and IFN-β-dependent proteins and protect epithelial cells in vitro from virally-induced cell death and to protect mice from a lethal dose of H1N1 influenza A virus. DMXAA that activates multiple antiviral pathways including IFN-β pathway is an attractive strategy in antiviral therapies. Nucleotide oligomerization domain 1 pathway may play an important role in refractoriness of IFN-β signaling in mouse respiratory epithelial cells induced by DMXAA.

Yu Z, Predina JD, Cheng G. Refractoriness of interferon-beta signaling through NOD1 pathway in mouse respiratory epithelial cells using the anticancer xanthone compound. *World J Biol Chem* 2013; 4(2): 18-29 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i2/18.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i2.18

INTRODUCTION

The influenza pandemic of 1918 occurred suddenly and killed over 50 million people worldwide to be followed by subsequent two major pandemic in 1957 and 1968[1,2]. The 2009 H1N1 influenza A pandemic initially raised similar concerns, but luckily, the pathogenicity of this virus was relatively less severe.

Two approaches currently available for use against influenza are antiviral drugs and vaccines. The use of antiviral drugs is the first line of defense against a new influenza pandemic. Four antiviral drugs, including zanamivir, oseltamivir, amantadine and rimantadine are currently approved by Food and Drug Administration in United States to treat acute, uncomplicated influenza. Although extremely valuable, their widespread use will likely be limited by concerns over side effects, drug resistance, *etc.*[2,3]. The best defense against influenza is vaccinated with appropriate vaccines. However, it is the most difficult defense to achieve. The 2009 pandemic of H1N1 influenza made it painfully clear how difficult it will be to generate vaccine quickly enough and in sufficient quantities for use during the initial stages of a pandemic. The need for new anti-influenza treatment or prophylactic strategies that can be employed rapidly is obvious.

To explore a different antiviral strategy by using a drug that activates antiviral innate immunity in a very rapid and non-specific fashion allowing for protection from any newly arising strain of influenza or other viral respiratory pathogen such as SARS will be important. The primary targets of respiratory viruses are respiratory tract mucosal epithelial cells, which form the majority of the cells lining the epithelial tract and lungs, and usually resistant to viral infection due to a complex defense system involving physical barriers, innate immune responses of the epithelial cells and resident leukocytes, and finally the development of acquired immune responses. One of the most important protections against viral infections is the release of a variety of immunostimulatory cytokines and chemokines by epithelial cells, macrophages and neutrophils, the most important being interferon (IFN)-α and IFN-γ[4]. These type I IFNs trigger the upregulation of a cascade of antiviral genes such as 2',5'-oligoadenylate synthetase 1 (OAS1) and myxovirus resistance 1 (Mx1) that protect cells from viral replication[5-7]. It has been reported that 5,6-di-methylxanthenone-4-acetic acid (DMXAA) can induce protection against vesicular stomatitis virus and H1N1 influenza A respiratory viral infections through innate immune activation, supported by inducing type I IFN signaling[8-13].

Despite much research that DMXAA can activate multiple innate immune pathways, the effects of DMXAA to activate IFN-β mediated antiviral signaling have never been studied in mouse thioglycollate-elicited peritoneal macrophages, bone marrow derived macrophages (BMDM) and bone marrow derived dendritic cells (BMDDC). Given its ability that type I IFN signaling became desensitization to DMXAA re-exposure in mouse macrophages cell line, we evaluated the hypothesis that DMXAA may also induce type I IFN signaling refractoriness in mouse respiratory epithelial cells. This was tested in the C10 mouse bronchial epithelial cells *in vitro* and nasal mucosa epithelial cells *in vivo*. Using siRNA targeting nucleotide oligomerization domain 1 (NOD1) or high-mobility group box 1/2/3 (HMGB1/2/3), we demonstrated that activation of IFN-β by DMXAA involved in NOD1 but not HMGB1/2/3 signal. The findings provide clear evidence that development of this approach, could offer an alternative therapeutic strategy in hosts refractory to IFN-β signaling or paralyzed by viral infection, may be especially valuable. This study also gives a hint that whether refractoriness also occurs during DMXAA treatment in lung cancer of clinical trials.

MATERAILS AND METHODS

Cell culture and reagents

C10 cells are a nontumorigenic murine alveolar type Ⅱ-like epithelial cell line that show the presence of lamellar bodies and the biosynthesis of surfactant[13]. Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin. Mouse thioglycollate-elicited peritoneal macrophages were collected and cultured as previously described[14]. The cells were maintained at 37 ℃ in an atmosphere containing 5%CO_2 and were regularly tested and maintained negative for Mycoplasma spp. Ultrapure lipopolysaccharide (LPS) was purchased from Invivogen (Carlsbad, CA, United States). The CpG oligodeoxynucleotide TCCATGACGTTCCTG ATGCT, known as CpG 1668, was synthesized on a phosphorothioate backbone (Integrated DNA technologies, Coralville, IA, United States).

Mice

Pathogen-free female BALB/c mice (6-8 wk old) were purchased from Charles River Laboratories (Wilmington, MA, United States). Animals were housed in the animal facility at the Wistar Institute (Philadelphia, PA, United States). The animal use committees of the Wistar Institute and University of Pennsylvania approve all protocols in compliance with the care and the use of animals[8,9,13].

Isolation and culture of mouse BMDM

After mice euthanized by CO_2 asphyxiation, pelvic and femoral bones of mice were removed. The marrow was flushed from the bones with 10 mL of culture medium and then centrifuged at 1000 rpm for 10 min at 4 ℃. Cells were resuspended in 10 mL L929 hybridoma (secreted macrophage colony-stimulating factor, M-CSF) conditioned medium after washed twice with Dulbecco's modified Eagle's medium plus 10%FBS and gently aspirated and expelled using needles until the cell aggregates broken up. Cells were incubated at 37 ℃ with 5%CO_2 in L929 conditioned medium for 1-2 wk. The BMDM were stained with CD11b and determined to be more than 92% pure macrophages by flow cytometry.

Generation of mouse BMDDC

BMDDC were cultured as previously described[9]. Briefly, in 24-well plates, 1×10^6 total bone marrow cells per well were seeded in 1 mL Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Carlsbad, CA, United States) with 10%FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L *L*-glutamine, 1.5 μmol/L 2-ME, 3 ng/mL granulocyte M-CSF and 3 ng/mL interleukin (IL)-4. After 7 d of culture, the cells were enriched using magnetic beads to a purity of at least greater than 93% $CD11c^+$ cells. Briefly, 1 μL biotinylated anti-CD11c antibodies (BD PharMingen) per 15×10^6 cells was added to cells suspended in at least 500 μL buffer comprised of PBS with 2 mmol/L EDTA and 0.5% bovine serum albumin. After 30 min at 4 ℃, cells were washed with buffer and labeled with MACS Streptavidin MicroBeads (Miltenyi Biotec) per manufacturer's instructions. Cells were then positively selected using MACS LS columns (Miltenyi Biotec) and washed.

Protein studies for cytokine/chemokine levels

The amount of IFN-inducible protein-10 (IP10/CXCL10), macrophage chemotactic protein (MCP1/CCL2) and IL-6 secreted by cells activated by DMXAA was quantified using enzyme-linked immunosorbent assay (ELISA) kits to detect murine IP10 (R and D Systems, Inc, Minneapolis, MN, United States), MCP1 and IL-6 (BD Biosciences Pharmingen, San Diego, CA, United States) according to the instructions of the manufacturers[12,13].

RNA isolation and real time, reverse transcription-polymerase chain reaction

Total RNA was isolated from cells or nasal epithelium with RNeasy Plus Mini Kit (Qiagen, Valencia, CA, United States), and cDNA was synthesized. Gene expression was checked using Applied Biosystems StepOne Real-time polymerase chain reaction (PCR) System following the manufacturer's protocol[12,13]. Primer sequences can be obtained from the authors on request.

Small interfering RNA

Transfection of siRNA control and NOD1 duplexed RNA oligonucleotides was performed using siRNA transfection reagent (Santa Cruz Biotechnology, Santa Cruz, CA, United States) according to the manufacturer's instructions[12]. After varying amounts of time, C10 cells were stimulated with DMXAA 100 μg/mL for 6 h, supernatants were collected for IP10 ELISA, and cells were harvested for RNA isolation. Transfection of siRNA control and HMGB1/2/3 siRNA (customized pan-HMGB siRNA, Santa Cruz Biotechnology, Santa Cruz, CA, United States) that targeting the sequence for 5'-GTA TGA GAA GGA TAT TGC T-3' was performed using siRNA transfection reagent (Santa Cruz Biotechnology, Santa Cruz, CA, United States)[12]. After amount of time, C10 cells were stimulated with DMXAA 100 μg/mL or Poly I-C 2 μg/mL for 6 h, then harvested for RNA isolation.

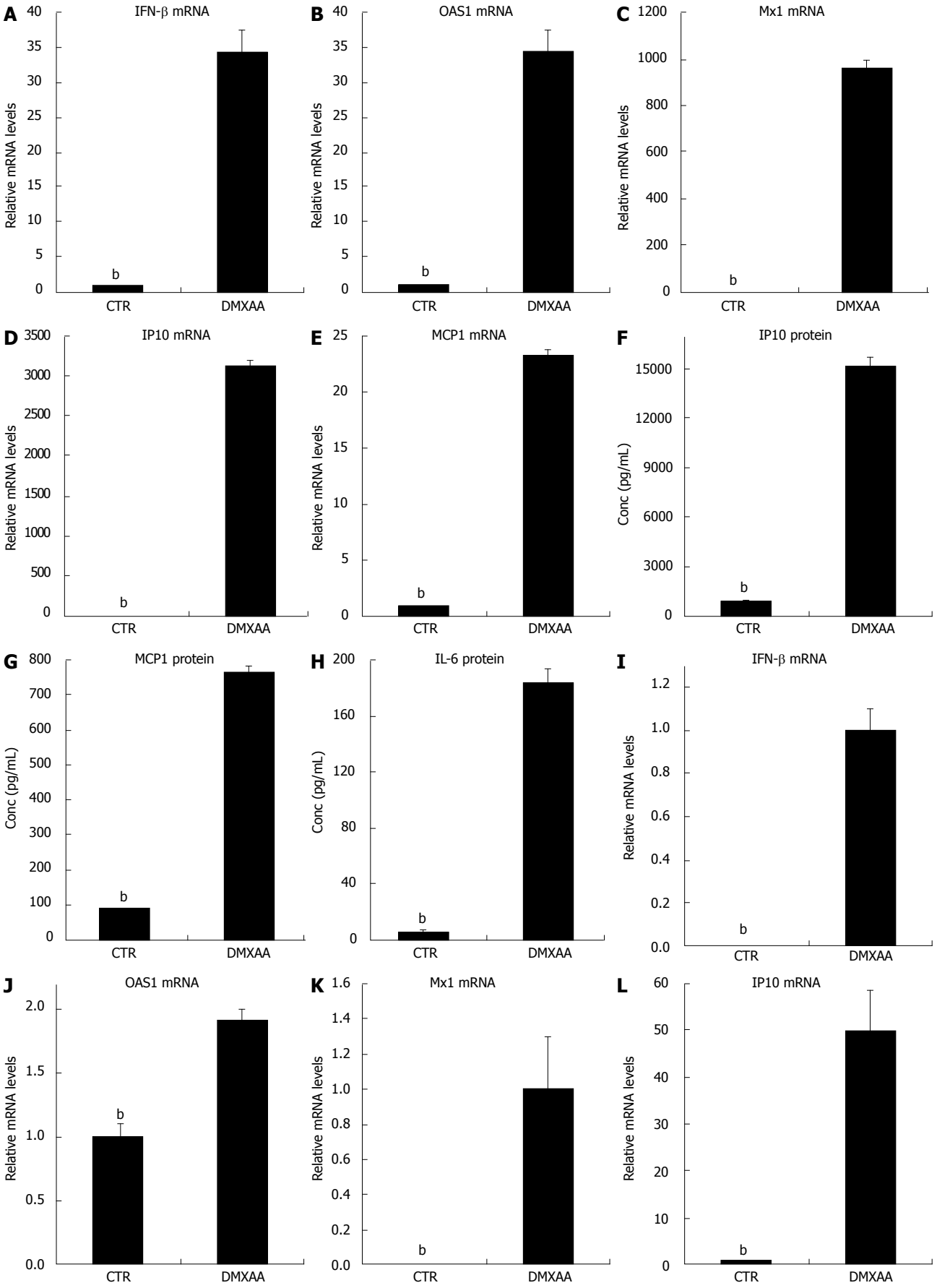
Statistical analysis

Unless otherwise noted, data comparing differences between two groups were assessed using unpaired Student's *t* test. Comparisons with more than two groups were done using one way ANOVA with appropriate post hoc testing. Differences were considered significant when $P < 0.05$. Data are presented as mean ± SE. Results are representative of two to three independent experiments.

RESULTS

DMXAA activates IFN-β-mediated antiviral signaling in mouse thioglycollate-elicited peritoneal macrophages and BMDM

Macrophages have a central role in innate immunity. To investigate the ability of DMXAA activation of IFN-β and IFN-γ responsive genes in different sources of macrophages, thioglycollate-stimulated peritoneal macrophages isolated by lavage from the peritonea of mice injected with thioglycollate for 3 d, cells were exposed to DMXAA. The mRNA expression profiles were evaluated using real time RT-PCR. IFN-β and IFN-γ mediated antiviral genes including OAS1 and Mx1 were significantly upregulated by DMXAA[13,15]. Marked and significant increases in IP10 and MCP-1 mRNA levels were also noted (Figure 1A-E). The concentrations of IP10, MCP1 and IL-6 proteins in the supernatants evaluated by ELISA, consistent with the mRNA data, were strongly elevated (Figure 1F-H). The mRNA levels of IFN-β OAS1 and Mx1, mRNA and protein levels of IP10 and MCP1, and protein level of IL-6 were significantly upregulated by DMXAA in thioglycollate-stimulated mouse peritoneal macrophages. To provide additional evidence for activation of IFN-β and IFN-γ mediated antiviral genes in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IFN-β mRNA
Relative mRNA levels
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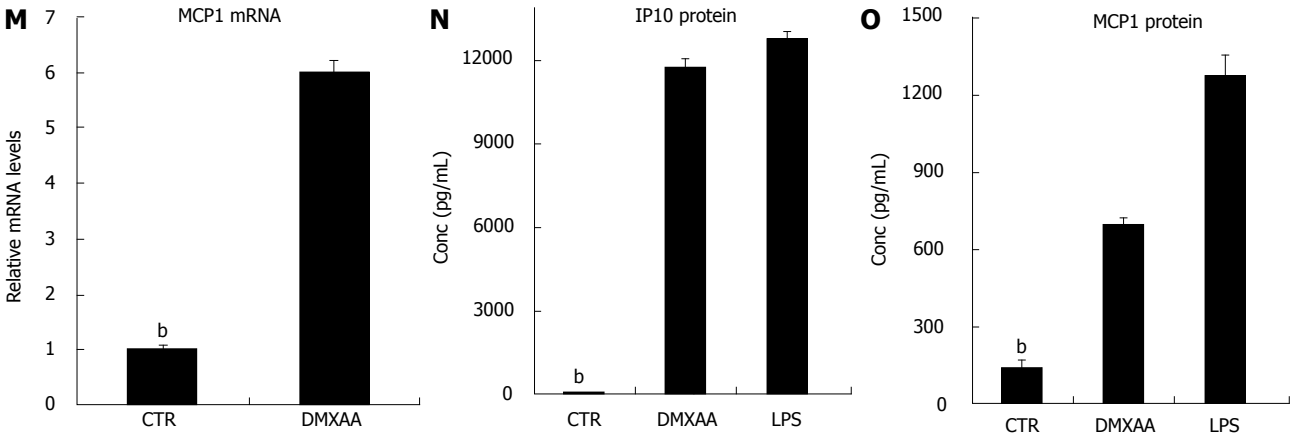

Figure 1 5,6-dimethylxanthenone-4-acetic acid elevates interferon-β-mediated antiviral genes and other chemokine/cytokine levels in mouse thioglycollate-elicited peritoneal and bone marrow derived macrophages. A-E: Cells were incubated with or without 5,6-dimethylxanthenone-4-acetic acid (DMXAA) 100 μg/mL for 5 h. The total RNA was isolated and the relative interferon (IFN)-β, 2',5'-oligoadenylate synthetase 1 (OAS1) and myxovirus resistance 1 (Mx1), inducible protein-10 (IP10), macrophage chemotactic protein (MCP1) and interleukin (IL)-6 mRNA levels were measured by real time reverse transcription-polymerase chain reaction; F-H: Induction of IP10, MCP1 and IL-6 protein was determined from the supernatant by enzyme-linked immunosorbent assay (ELISA); I-M: Cells were incubated with or without DMXAA 100 μg/mL or lipopolysaccharide (LPS) 100 ng/mL for 5 h. The total RNA was isolated and the relative IFN-β, OAS1 and Mx1, IP10, MCP1 and IL-6 mRNA levels were measured by real time reverse transcription-polymerase chain reaction; N,O: Induction of IP10 and MCP1 protein was determined from the supernatant by ELISA. The results presented as mean ± SE ($^bP < 0.01$ *vs* DMXAA or LPS).

macrophages by DMXAA, BMDM were exposed to DMXAA or TLR4 ligand LPS. IFN-β and IFN-γ mediated antiviral genes *OAS1* and *Mx1* were significantly increased by DMXAA (Figure 1I-M). IP10 and MCP-1 inflammatory cytokine/chemokine mRNA levels were also significant upregulated by DMXAA. Protein levels of IP10 and MCP1 in the supernatants evaluated by ELISA were significantly upregulated by DMXAA or LPS in BMDM (Figure 1N and O). The data presented here indicated that DMXAA activated IFN-β and IFN-γ mediated antiviral genes *OAS1*, *Mx1*, inflammatory cytokine/chemokine IP10 and MCP-1 in BMDM. LPS activated inflammatory cytokine/chemokine IP10 and MCP-1 in BMDM.

DMXAA activates IFN-β mediated antiviral signaling in mouse BMDDC

Dendritic cells are antigen-presenting cells that process antigen materials and present them on the surface to other cells of the immune system function as messengers between the innate and adaptive immunity. Our previous data have shown that DMXAA can directly activate mouse BMDDC inducing proinflammatory cytokine production and costimulatory molecule expression *in vitro* in a MyD88-independent fashion. Next, we investigate whether DMXAA can effectively activate IFN-β and IFN-γ responsive genes in mouse BMDDC, cells were exposed to DMXAA, LPS and TLR9 agonist CpG DNA, respectively[9]. DMXAA, LPS or CpG DNA caused rapid, and in some cases, very large increases in IFN, IFN-β mediated antiviral genes *OAS1*, *Mx1*, inflammatory cytokine/chemokine IP10 and MCP-1 (Figure 2). CpG DNA did not induce significantly increase of IFN-β and OAS1 in mouse BMDDC (Figure 2, $P > 0.05$).

DMXAA induces refractoriness of IFN-β signaling in C10 mouse respiratory epithelial cells

Given the activity that DMXAA activates IFN-β mediated antiviral signaling pathways in C10 cells[13], and DMXAA pretreatment of macrophage cell line induces a state of refractoriness to re-exposure DMXAA[11], the response of C10 cells to re-exposure DMXAA was examined. C10 cells were pretreated with DMXAA. After 72 h, cells were continued to incubate with DMXAA for 3 h (DMXAA 75 h), cells were washed and re-exposure to fresh DMXAA in the same condition for 3 h (re-DMXAA 3 h). C10 cells stimulated with DMXAA for 3 h were set up as positive control. Analysis of the response of cells to DMXAA revealed a strong increase in IP10 mRNA (Figure 3A) at 3 h. DMXAA upregulated IP10 mRNA declined at 75 h (Figure 3B), though the level of IP10 mRNA was still strongly and persistently elevated. DMXAA rechallenged for 3 h induced very little IP10 mRNA (Figure 3B, 4 fold over medium control, $P < 0.01$) *vs* DMXAA 3 h incubation (Figure 3A). Similar to IP10 mRNA level, DMXAA significantly induced RANTES mRNA (Figure 3C) at 3 h. DMXAA upregulated RANTES mRNA declined at 75 h (Figure 3D, 4 fold over medium control, $P < 0.01$). DMXAA rechallenged for 3 h induced some RANTES mRNA (Figure 3D, 5 fold over medium control, $P < 0.01$) *vs* DMXAA 3 h of incubation (Figure 3C). Moreover, DMXAA significantly upregulated IFN-β mRNA (Figure 3E) at 3 h of incubation. Quite striking and unexpected, DMXAA induced a diminished response in IFN-β mRNA expression, DMXAA upregulated IFN-β mRNA declined at 75 h of incubation (Figure 3F). DMXAA rechallenged for 3 h downregulated IFN-β mRNA level (Figure 3F) *vs* DMXAA 3 h of incubation (Figure 3E). DMXAA rechallenged for 3 h stimulated

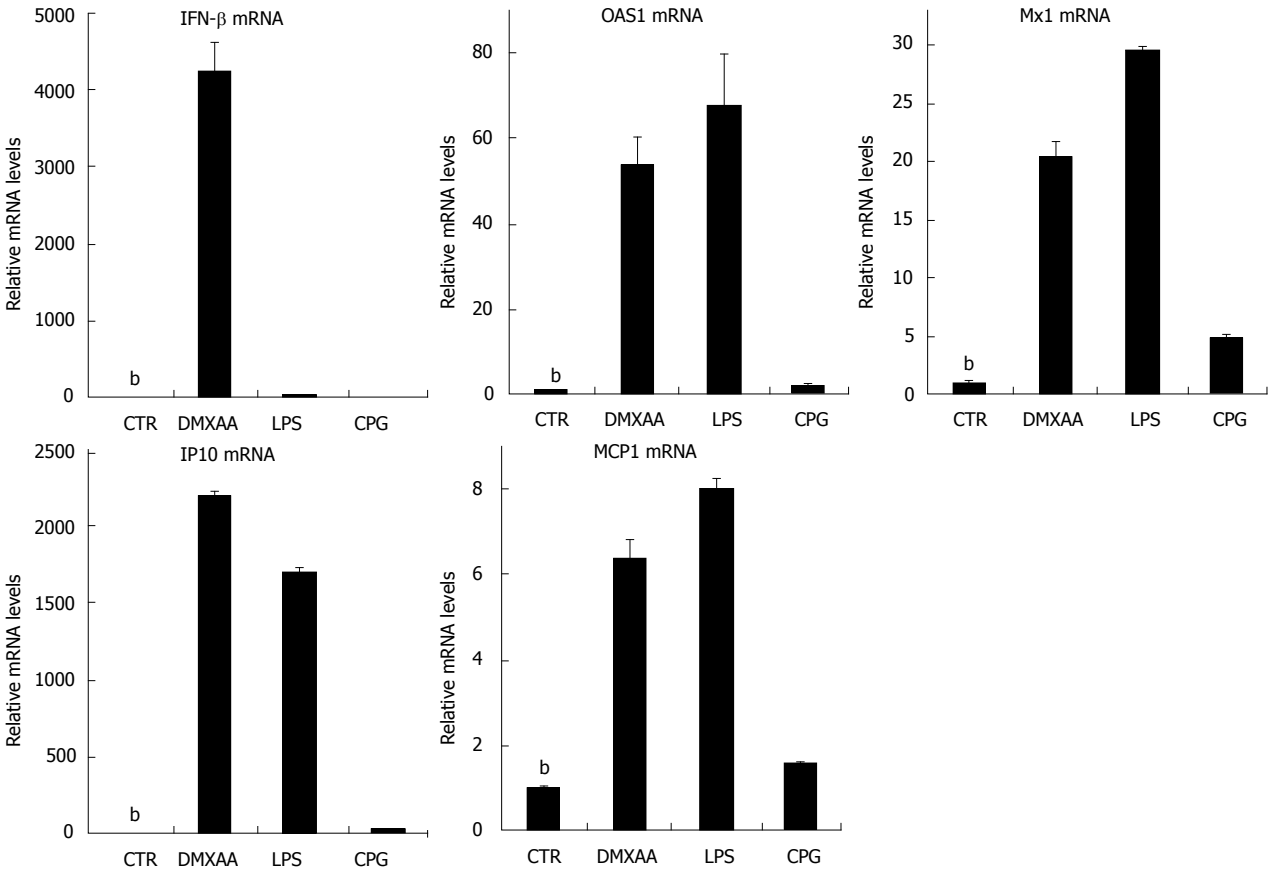

Figure 2 5,6-dimethylxanthenone-4-acetic acid activates interferon-β-mediated antiviral genes and other chemokine/cytokine levels in mouse bone marrow derived dendritic cells. Cells were incubated with or without 5,6-dimethylxanthenone-4-acetic acid (DMXAA) 100 μg/mL, lipopolysaccharide (LPS) 100 ng/mL and CpG DNA 0.5 μmol/L for 6 h, respectively. The total RNA was isolated and the relative interferon (IFN)-β, 2',5'-oligoadenylate synthetase 1 (OAS1) and myxovirus resistance 1 (Mx1), inducible protein-10 (IP10), macrophage chemotactic protein (MCP1) mRNA levels were measured by real time reverse transcription-polymerase chain reaction. The results presented as mean ± SE ($^bP < 0.01$ *vs* DMXAA, LPS and CpG DNA, respectively).

a diminished response in IFN-β mRNA expression indicated that some refractoriness was induced. It could be explained by hypo-response of the signal pathway. Interestingly, DMXAA significantly upregulated NOD1 mRNA (Figure 3G) at 3 h of incubation. DMXAA increased NOD1 mRNA declined at 75 h of incubation (Figure 3H). DMXAA rechallenged for 3 h induced some refractoriness in NOD1 mRNA expression (Figure 3H) *vs* medium control. It is hypothesized that refractoriness of NOD1 mRNA could explain IFN-β refractory signal both induced by 75 h of incubation and rechallenged for 3 h of DMXAA.

DMXAA induces refractoriness of IFN-β signaling in mouse mucosa epithelial cells

To examine whether refractoriness of IFN-β signaling occurs *in vivo*, mice were given DMXAA intranasally. After 7 d, mice were rechallenged with same dose of DMXAA intranasally for 3 h (re-DMXAA 3 h group) or mice were continued to wait for 3 h (DMXAA 7 d + 3 h group). Another group of naïve mice administrated DMXAA intranasally for 3 h was set up as positive control (DMXAA 3 h group). Mice were sacrificed and nasal epithelia were removed[13]. Mice pretreatment with DMXAA for 7 d still had strong response to secondary DMXAA administration for 3 h with significant 14.5 fold increase in IFN-β mR expression, similar to mice administrated one dose DMXAA for 3 h with strong increase in IFN-β mR level (Figure 4) DMXAA significantly upregulated OAS1, Mx1, IP10 and NOD1 mRNA levels in DMXAA 3 h group of one dose administration. DMXAA rechallenged 3 h group still had strong OAS1, Mx1, IP10 and NOD1 mRNA expression (Figure 4), but significately declined compared to that of DMXAA 3 h group with one dose administration. No significant differences were observed in mRNA levels of IFN-β, OAS1, Mx1, IP10 and NOD1 between DMXAA rechallenged 3 h group and DMXAA 7 d + 3 h group (data not shown). The results indicated that pretreatment with DMXAA for 7 d, did not induce strong refractoriness to secondary DMXAA administration *in vivo* (Figure 4).

Role of NOD1 and HMGB1/2/3 pathways in DMXAA induced IFN-β signaling

We wanted to demonstrate that DMXAA could activate IFN-β production *via* the NOD1 signal. C10 cells were transfected with NOD1 siRNA. NOD1 siRNA was highly effective in down regulating baseline levels

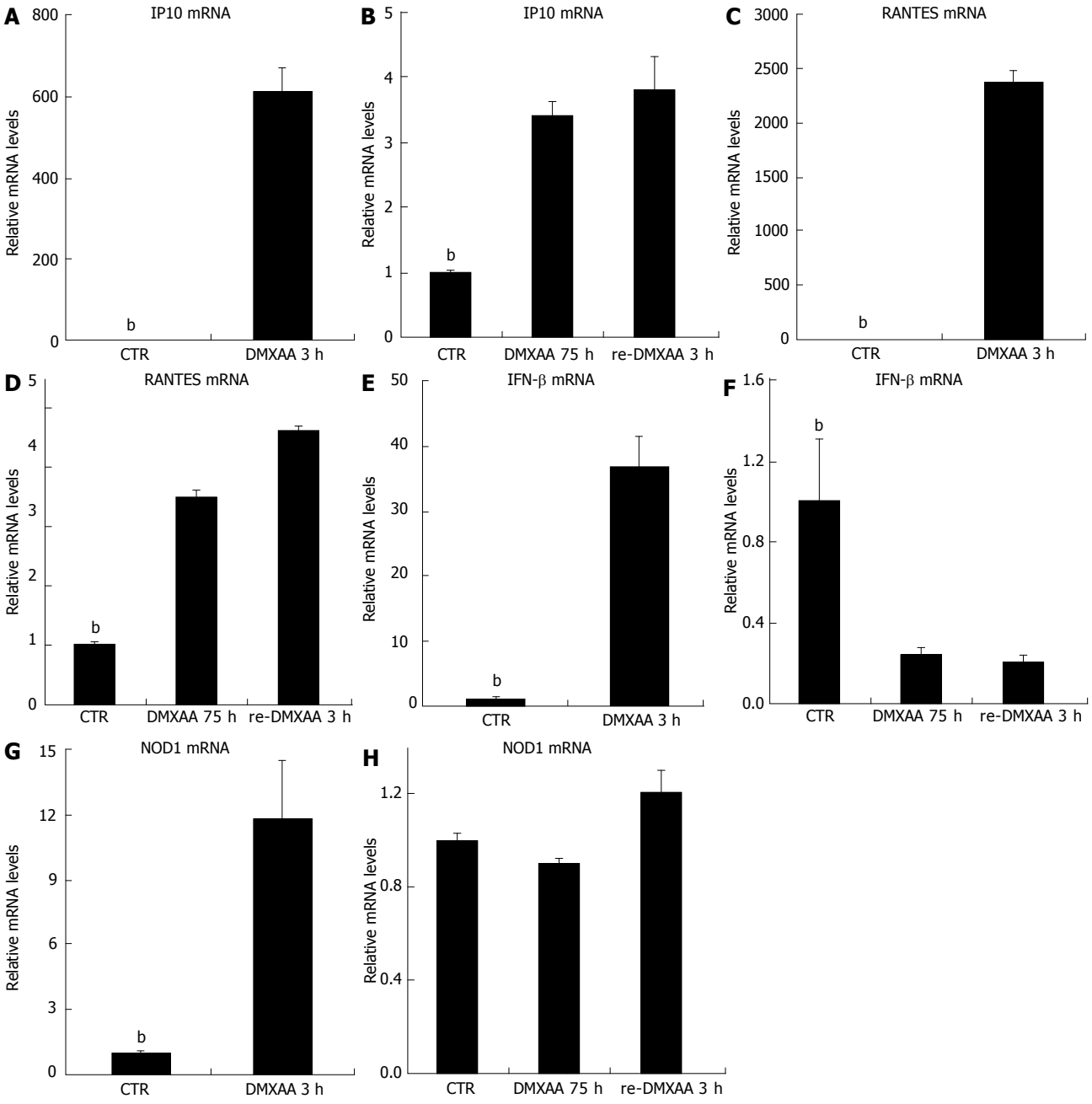

Figure 3 5,6-dimethylxanthenone-4-acetic acid pretreatment of C10 mouse bronchial epithelial cells induces refractoriness to secondary 5,6-dimethylxanthenone-4-acetic acid administration. Cells were rechallenged with same concentration of 5,6-dimethylxanthenone-4-acetic acid (DMXAA) 3 h after pretreatment with DMXAA 100 μg/mL for 72 h. The total RNA was isolated and the relative inducible protein-10 (IP10) (A, B), Rantes (C,D), interferon (IFN)-β (E, F) and nucleotide oligomerization domain 1 (NOD1) (G,H) mRNA levels were measured by real time reverse transcription-polymerase chain reaction. The results presented as mean ± SE ($^bP < 0.01$ *vs* DMXAA groups).

of NOD1 mRNA at 24 and 72 h time points (Figure 5A). C10 cells were thus treated with NOD1 siRNA or control siRNA and then exposed to DMXAA[12]. Interestingly, DMXAA markedly upregulated NOD1 mRNA, however the NOD1 siRNA clearly blunted this increase in NOD1 message (Figure 5B). Importantly, knockdown of NOD1 mRNA significantly inhibited the response of C10 cells to DMXAA, mRNA levels of IFN-β and IP10 were significantly reduced (Figure 5C and D) and secreted level of IP10 protein was reduced by 40% after exposure to DMXAA (Figure 5E). This partial response could be due to incomplete knockdown of NOD1 activity, or DMXAA stimulation of other pathways. The results indicated that NOD1 is required for IFN-β and IP10 signals in response to DMXAA induction. HMGB functions as an universal sentinel in type I IFN signaling and inflammatory cytokine induction by DNA or RNA targeted to activate the cytosolic nucleic-acid sensing receptors. We therefore wanted to demonstrate whether DMXAA activates IFN-β signal *via* the HMGB pathway. C10 cells were transfected with HMGB1/2/3 siRNA. HMGB1/2/3 siRNA was highly effective in down regulating baseline levels of HMGB1 (Figure 6A), HMGB2 (Figure 6B) and HMGB3 (Figure 6C), respectively at 48

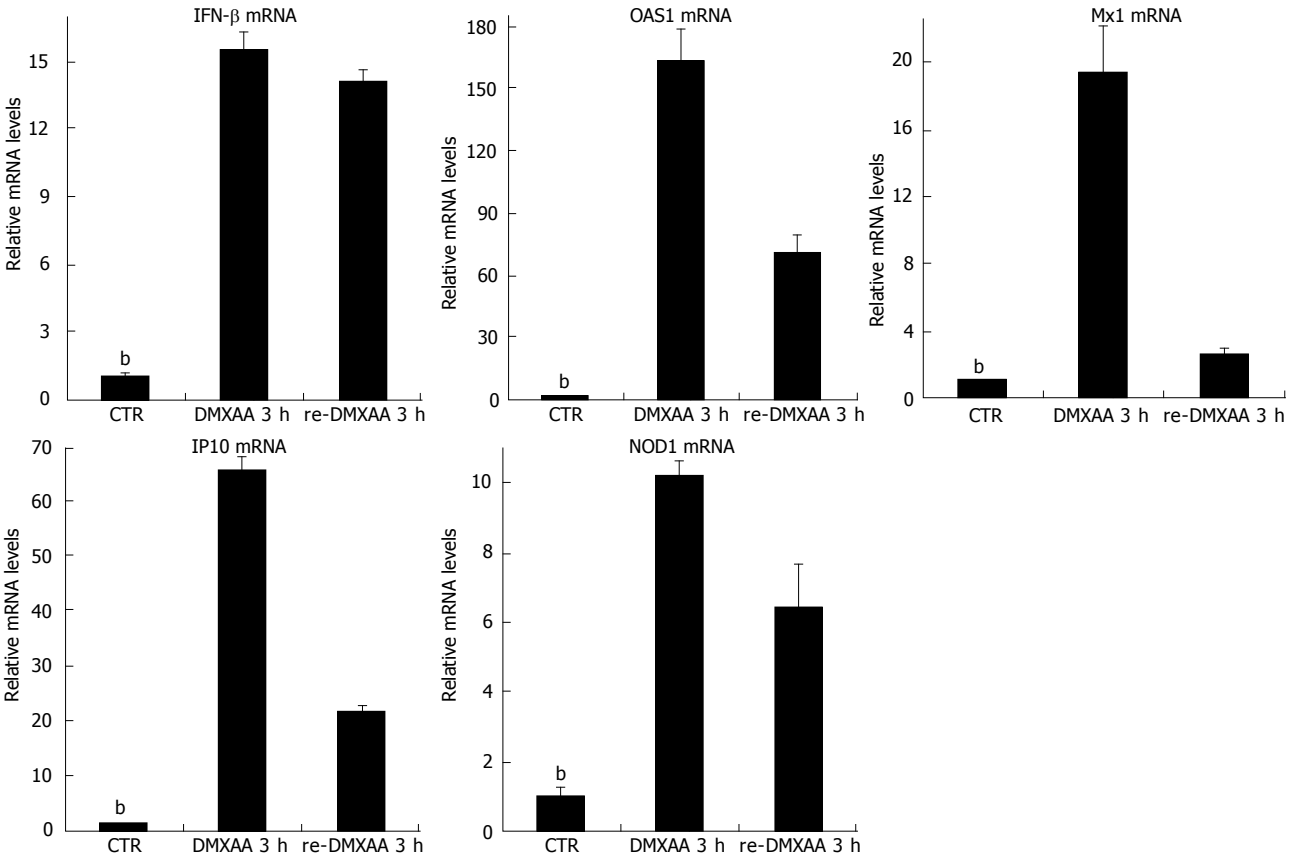

Figure 4 5,6-dimethylxanthenone-4-acetic acid induces refractoriness of interferon-β signaling *in vivo*. Mice were rechallenged with 5,6-dimethylxanthenone-4-acetic acid (DMXAA) 3 h after pretreatment with DMXAA for 7 d, relative interferon (IFN)-β, 2',5'-oligoadenylate synthetase 1 (OAS1) and myxovirus resistance 1 (Mx1), inducible protein-10 (IP10), and nucleotide oligomerization domain 1 (NOD1) mRNA levels of nasal epithelia were measured by real time reverse transcription-polymerase chain reaction. The results presented as mean ± SE ($^bP < 0.01$ *vs* DMXAA group, 5 mice each group).

h. Cells were then treated with HMGB1/2/3 siRNA or control siRNA for 48 h, and furthermore exposed to DMXAA or TLR3 ligand Poly I-C (positive control) for 6 h. Both DMXAA and Poly I-C markedly upregulated IFN-β mRNA, however, the HMGB1/2/3 siRNA did not blunted this increase of DMXAA induced IFN mRNA. Importantly, knockdown of HMGB1/2/3 significantly inhibited the response of cells to Poly I-C induced IFN mRNA (Figure 6D, $P < 0.05$). The knockdown expression profiles suggest an involvement of HMGB signal in induction of IFN-β mRNA by Poly I-C, but not by DMXAA. DMXAA induced IFN-β mRNA through stimulation of other pathways, such as NOD.

DISCUSSION

Initial studies suggested that DMXAA, directly activate innate immune system, can be used to protect from influenza pneumonia[13]. Desensitization of IFN-β signal in mouse macrophage cell line on re-exposure to DMXAA has been demonstrated years ago[11]. Up to now, it is little to know whether or to what degree of IFN-β signaling refractoriness is induced by DMXAA re-administration in mouse respiratory epithelial cells. The studies presented here thus identify DMXAA has ability to activate IFN-β dependent antiviral gene and other chemokine/cytokine expressions in mouse thioglycollate-elicited peritoneal macrophages, BMDM and BMDDC (Figures 1 and 2). Pretreatment with DMXAA induces IFN-β siganling refractoriness in respiratory epithelial cells *in vitro* and *in vivo*. NOD1 but not HMGB1/2/3 involved in IFN-β signal refractoriness induced by DMXAA re-exposure in mouse respiratory epithelial cells (Figures 3-6). The data indicated that IFN-β signal refractoriness may also happen in clinical administration of DMXAA and reduce the therapeutic efficacy, but no direct clinical observations.

Viral infection activates a variety of pattern recognition-receptors (PRR), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) that induce the production of IFN regulatory factor 3 (IRF3), nuclear factor-κB (NF-κB), and other pathways[15,16]. Virally-induced secretion of IFN-β largely mediated through IRF3 is especially important, as this molecule then acts in a paracrine fashion on neighboring cells to upregulate a group of key antiviral proteins (such as OAS1, Mx1 and protein kinase R) that prevent subsequent infection and spread of the virus. Most viruses have developed sophisticated and different mechanisms to try prevention IFN pathway activation[13,17,18]. An alternative strategy to harness the innate immune system is to use agents that mimic viral infection and activate the innate immunity through PRR. Type I IFNs are essential for host defenses against vi-

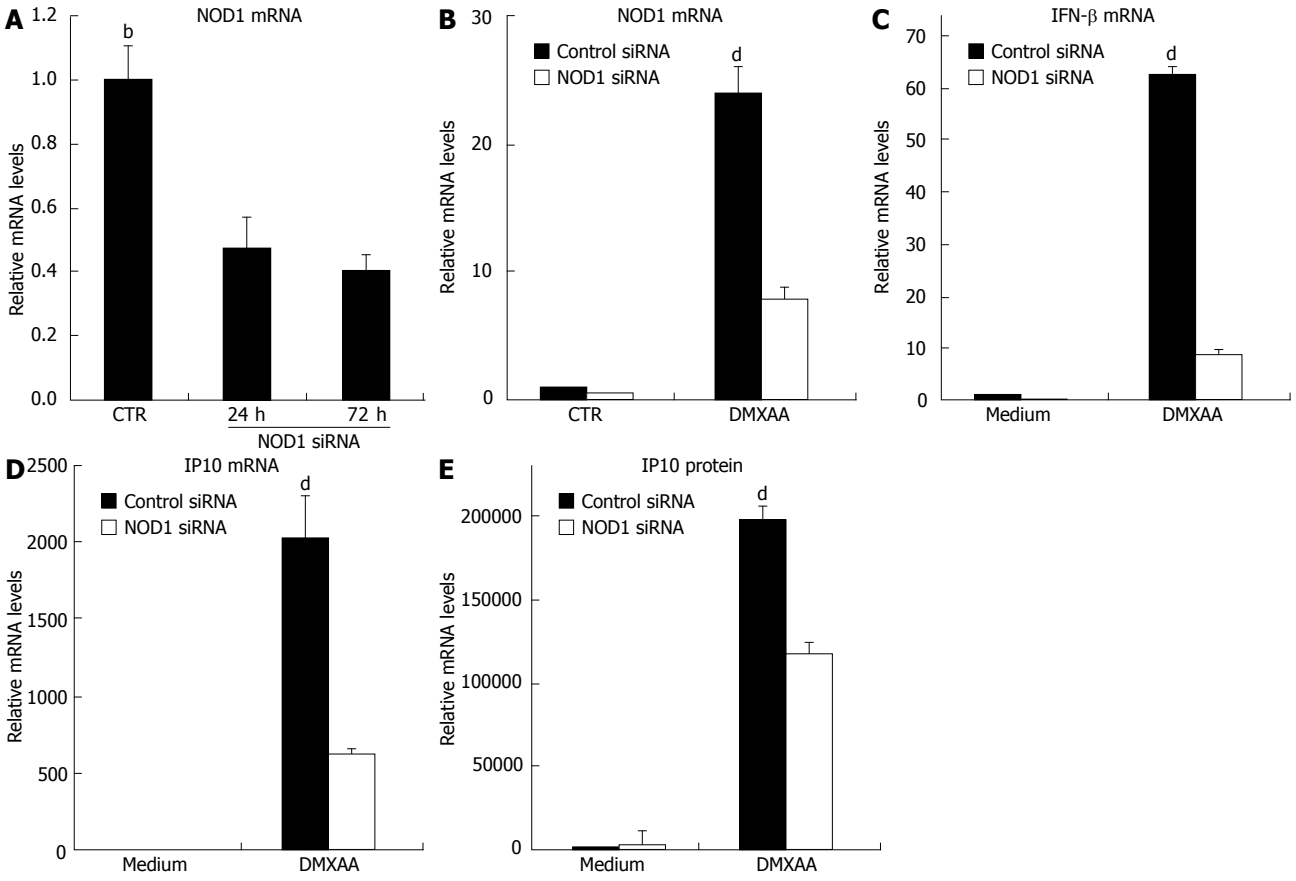

Figure 5 5,6-dimethylxanthenone-4-acetic acid-induced interferon-β expression inhibited by nucleotide oligomerization domain 1 small interfering RNA. A: C10 cells were transfected with control small interfering RNA (siRNA) or nucleotide oligomerization domain 1 (NOD1) siRNA. Total RNA was extracted at indicated time points and the relative NOD1 mRNA levels were measured by real time real time reverse transcription-polymerase chain reaction (RT-PCR). After transfected with control siRNA or NOD1 siRNA for 72 h, C10 cells were further incubated with or without 5,6-dimethylxanthenone-4-acetic acid 100 μg/mL for 6 h; B-E: The relative NOD1, interferon (IFN)-β and inducible protein-10 (IP10) mRNA levels were measured by real time RT-PCR or the supernatants were removed and IP10 levels were measured by enzyme-linked immunosorbent assay ($^{b}P < 0.01$ *vs* 24 h or 72 h; $^{d}P < 0.01$ *vs* NOD1 siRNA, data are shown as the mean ± SE).

ruses. As early as the 1960s, type I IFNs were reported to be antivirals[19-23]. Refractoriness to IFN within h and continuous up to 3 d in cultured human fibroblasts was observed[24,25]. Four known TLRs (TLRs 3, 7, 8 and 9) recognize different forms of "foreign" nucleic acids, and induce IFN to activate antiviral genes[26]. Compared to using recombinant IFN protein, agonists that activate specific TLRs have been shown to be less toxic, are easier to administer and more effective[27]. TLR3 agonist Poly I-C has been studied for the treatment of influenza infection[28]. TLR4, TLR7 and TLR9 ligands, including LPS, single-stranded RNA and CpG-rich DNA, respectively, triggered IFN induction in cultured cells[29]. Studies reported that TLR ligands can be used against influenza infection but with some major limitations as antiviral agents.

In this study, we showed that DMXAA stimulated IFN-β production and IFN-β-dependent antiviral gene *OAS1*, *Mx1* and chemokine/cytokine IP10, *MCP1* and *IL-6* expressions in mouse thioglycollate-elicited peritoneal macrophages, BMDM and BMDDC. To our knowledge, this is the first report that DMXAA can stimulate IFN-β dependent antiviral gene *OAS1* and *Mx1* expressions in mouse macrophages and dendritic cells. Macrophages and dendritic cells function as major sensors of invading pathogens[30]. It has been shown that TLRs are not involved in activation of dendritic cells of DMXAA induction[9].

To determine whether there is any induction of refractoriness by DMXAA on IFN relative genes, C10 cells were stimulated with DMXAA (Figure 3). DMXAA re-exposure induced refractoriness that was characterized by an almost complete inhibition of IFN-β expression (Figure 3B, D, F, and H). We next showed that the recovery of refractoriness of IFN mRNA is essentially complete about 7 d *in vivo*, a little different profiles from that of OAS1, Mx1 and IP10 mRNA levels (Figure 4).

NOD1, a member of the NLR family which are intracellular cytoplasmic sensors, is widely expressed in mammals but seems to be especially important in epithelial and mesothelial cells[12]. DMXAA activates NOD rip-like interacting caspase-like apoptosis-regulatory protein kinase (RICK) pathway[12]. We wanted to evaluate whether DMXAA-induced NOD activation involved in refractoriness of IFN-β signaling. It was able to show inhibition of DMXAA-induced IFN-β, IP10 mRNA and/or protein

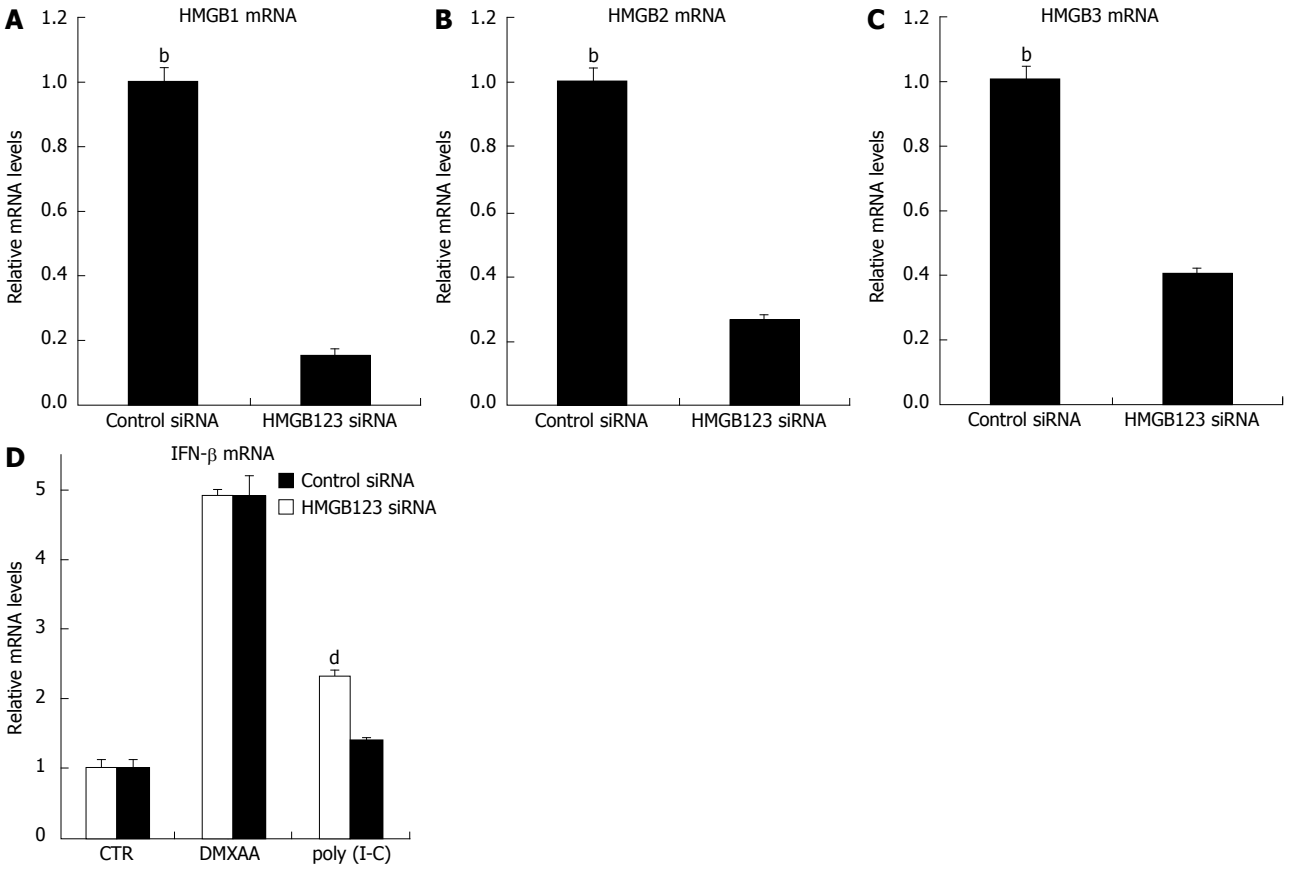

Figure 6 High-mobility group box 1/2/3 pathway did not involved in 5,6-dimethylxanthenone-4-acetic acid-induced interferon-β expression. A-C: C10 cells were transfected with control small interfering RNA (siRNA) or high-mobility group box 1/2/3 (HMGB1/2/3) siRNA, the relative of each HMGB mRNA level was measured by real time reverse transcription-polymerase chain reaction (RT-PCR), respectively, HMGB1, HMGB2 and HMGB3; D: C10 cells were transfected with control siRNA or HMGB1/2/3 siRNA, then cells were further incubated with or without DMXAA or Poly I-C. The relative interferon (interferon) mRNA levels were measured by real time RT-PCR ($^{b}P < 0.01$ *vs* HMGB1/2/3 siRNA; $^{d}P < 0.01$ *vs* HMGB1/2/3 siRNA in Poly I-C group).

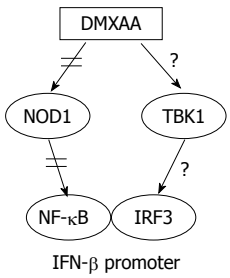

Figure 7 Schematic model of probable pathway of refractoriness of interferon-β signal. Previous works have shown that 5,6-dimethylxanthenone-4-acetic acid (DMXAA) can activate TANK-binding kinase1 (TBK1) to initiate interferon (IFN) regulatory factor 3 (IRF3) signaling and nucleotide oligomerization domain 1 (NOD1) that interacts with rip-like interacting caspase-like apoptosis-regulatory protein kinase to activate nuclear factor-κB (NF-κB) signal pathway that regulates production of inflammatory cytokines/chemokines[11-13]. Our results indicate that DMXAA can induce refractoriness of interferon-β signal through NOD1 tolerance in mouse respiratory epithelial cells.

using siRNA to NOD1 (Figure 5B-E), but not DMXAA-induced IFN-β mRNA with siRNA to HMGB1/2/3 (Figure 6D). Activation of IFN-β by DMXAA involved in NOD1 but not HMGB1/2/3 signal pathway (Figures 4-6). We thus believe that DMXAA re-exposure induced refractoriness of IFN-β signal through NOD1 pathway (Figure 7). NOD1 mediates NF-κB activation required for IFN-β expression, but not IRF3[31]. This suggests that in pharmacological condition of DMXAA administration, there is another PRR functioning in tandem with NOD1 that involves in activation of IRF3 pathway[11,13,31]. It is not known for certain how important the NOD1 pathway *vs* other potential activating pathway in refractoriness of IFN-β signal. As one of the main cellular segregation and degradation systems, autophagy has recently caught the high attention in the fields of innate immunity. NOD1 and NOD2 as the link between intracellular bacterial sensing and induction of autophagy was reported[32]. It was unclear whether autophagy machinery involved in the effect of DMXAA. Determining where and how DMXAA induces autophagy in protection against virus infection and the relationship of autophagy and refractoriness of type I IFN signaling is an area of great interest[12,13].

There are a number of implications of this study. DMXAA shares virus-triggered innate immune pathway, at least including IFN-β, NOD/RICK and NF-κB signals. DMXAA had a relatively window of function and induced refractoriness of IFN-β signal. This suggests

that daily administration of such compounds like DMXAA, may not be effective as dosing intervals shorter than the period of refractoriness would strongly reduce the efficacy of the administration, that targeted signal pathways still in refractoriness.

In summary, DMXAA that activates multiple antiviral pathways including IFN-β pathway is an attractive strategy in antiviral therapies. Development of analogues of xanthone-like DMXAA, small molecular IFN-β inducing drugs without or less refractoriness of IFN-β signaling, to target specific IFN-β mediated antiviral pathways is much promising at treating particular virus.

ACKNOWLEDGMENTS

We thanks Dr. Steven M. Albelda for generous supports.

COMMENTS

Background

Drugs that can rapidly inhibit respiratory infection from influenza or other respiratory pathogens are needed. We recently demonstrated that a small, cell-permeable compound, 5,6-di-methylxan-thenone-4-acetic acid (DMXAA), was able to induce production of interferon (IFN)-γ and IFN-β-dependent proteins and protect epithelial cells *in vitro* from virally-induced cell death and to protect mice from a lethal dose of H1N1 influenza A virus. However, within hours of incubation, type I IFN signaling in mouse macrophage cell line was desensitization to DMXAA. In this study, authors assessed the possibility that DMXAA induced refractoriness of interferon-signaling involved in nucleotide oligomerization domain 1 (NOD1) pathway.

Research frontiers

One of the most important protections against viral infections is the release of a variety of immunostimulatory cytokines and chemokines by epithelial cells, macrophages and neutrophils. Viral infection activates a variety of pattern recognition-receptors, such as Toll-like recepters and NOD-like receptors, NOD-like receptors (NLRs). NOD1, a member of the NLR family which are intracellular cytoplasmic sensors, is widely expressed in mammals cells. DMXAA activates NOD/rip-like interacting caspase-like apoptosis-regulatory protein kinase pathway. The authors evaluated whether DMXAA-induced NOD activation involved in refractoriness of IFN-β signaling.

Innovations and breakthroughs

DMXAA takes advantage of the primary innate immune defense against viral infection by directly activating the interferon pathway. In this study, DMXAA activates IFN-β pathway with high level of IFN-β dependent antiviral genes in mouse thioglycollate-elicited peritoneal macrophages, bone marrow derived macrophages and bone marrow derived dendritic cells. Activation of IFN-β by DMXAA involved in NOD1 but not high-mobility group box 1/2/3 signal. NOD1 pathway plays an important role in refractoriness of IFN-β signaling induced by DMXAA in mouse C10 respiratory epithelial cells and BALB/c mice nasal epithelia. These data indicate that DMXAA is not well adapted to the intrinsic properties of IFN-β signaling.

Application

Approaches to restore sensitivity of IFN-β signaling by find other xanthone compounds may function similarly, could enhance the efficacy of protection from influenza pneumonia and potentially in other respiratory viral infections.

Peer review

This manuscript is very good for publication with minor revision. The drug refractoriness of anti-viral drugs is a very important topic, and the findings presented in this manuscript are generally interesting.

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P- Reviewers Clements JE, Ding WX
S- Editor Gou SX **L- Editor** A **E- Editor** Lu YJ

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
wjbc@wjgnet.com
doi:10.4331/wjbc.v4.i2.30

World J Biol Chem 2013 May 26; 4(2): 30-34
ISSN 1949-8454 (online)

BRIEF ARTICLE

Caspase-1 activation and mature interleukin-1β release are uncoupled events in monocytes

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Supported by NIH R21 AI085416 (to Shi J) and NIH NCRR P20-RR017686 (to PI: Daniel Marcus; Shi J)
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Received: February 13, 2013 Revised: March 26, 2013
Accepted: April 10, 2013
Published online: May 26, 2013

Abstract

AIM: To investigate whether caspase-1 activation/intracellular processing of pro-interleukin-1β (pro-IL-1β) and extracellular release of mature IL-1β from activated monocytes are separable events.

METHODS: All experiments were performed on fresh or overnight cultured human peripheral blood monocytes (PBMCs) that were isolated from healthy donors. PBMCs were activated by lipopolysaccharide (LPS) stimulation before being treated with Adenosine triphosphate (ATP, 1 mmol/L), human α-defensin-5 (HD-5, 50 μg/mL), and/or nigericin (Nig, 30 μmol/L). For each experiment, the culture supernatants were collected separately from the cells. Cell lysates and supernatants were both subject to immunoprecipitation with anti-IL-1β antibodies followed by western blot analysis with anti-caspase-1 and anti-IL-1β antibodies.

RESULTS: We found that pro-IL-1β was processed to mature IL-1β in LPS-activated fresh and overnight cultured human monocytes in response to ATP stimulation. In the presence of HD-5, this release of IL-1β, but not the processing of pro-IL-1β to IL-1β, was completely inhibited. Similarly, in the presence of HD-5, the release of IL-1β, but not the processing of IL-1β, was significantly inhibited from LPS-activated monocytes stimulated with Nig. Finally, we treated LPS-activated monocytes with ATP and Nig and collected the supernatants. We found that both ATP and Nig stimulation could activate and release cleaved caspase-1 from the monocytes. Interestingly, and contrary to IL-1β processing and release, caspase-1 cleavage and release was not blocked by HD-5. All images are representative of three independent experiments.

CONCLUSION: These data suggest that caspase-1 activation/processing of pro-IL-1β by caspase-1 and the release of mature IL-1β from human monocytes are distinct and separable events.

Key words: Caspase-1; Human defensin; Monocytes; Interleukin-1β processing and release; Inflammasome

Core tip: Activated macrophages release large amounts of interleukin-1β (IL-1β) and macrophages deficient in caspase-1 expression have undetectable IL-1β secretion. This suggests that IL-1β release and caspase-1 activation are closely related events. We found that human α-defensin 5 (HD-5) inhibited the release of IL-1β, but not the processing of pro-IL-1β to IL-1β in lipopolysaccharide-activated monocytes stimulated with Adenosine triphosphate or nigericin. Different from IL-1β processing

and release, the activation and release of caspase-1 from stimulated monocytes was not blocked by HD-5. These data suggest that caspase-1 activation/processing of pro-IL-1β by caspase-1 and the release of mature IL-1β from human monocytes are distinct and separable events.

Galliher-Beckley AJ, Lan LQ, Aono S, Wang L, Shi J. Caspase-1 activation and mature interleukin-1β release are uncoupled events in monocytes. *World J Biol Chem* 2013; 4(2): 30-34 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i2/30.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i2.30

INTRODUCTION

Interleukin-1β (IL-1β) is an important acute response factor of host defense against microbial infections and a key mediator of inflammation in multiple organs[1,2]. Viral and bacterial pathogens trigger inflammasome formation and subsequent caspase-1 activation and IL-1β maturation[3]. IL-1β is synthesized as a biologically inactive 31 kDa pro-IL-1β polypeptide and must be post-translationally processed by caspase-1 to generate the mature 17 kDa pro-inflammatory cytokine IL-1β[4]. Overproduction of IL-1β is associated with multiple autoimmune diseases and septic shock and animals deficient in IL-1β are highly susceptible to microbial infections[5,6].

Activated monocytes and macrophages rapidly release large amounts of mature IL-1β and inflammasome components including caspase-1[7]; macrophages deficient in caspase-1 expression have impaired processing of pro-IL-1β and undetectable IL-1β secretion[8]. This suggests that IL-1β release and caspase-1 activation are closely related events. Because most known inhibitors of IL-1β production block caspase-1 activation, previous studies are not able to determine whether caspase-1 activation/pro-IL-1β processing and IL-1β release are separate or linked processes. We have previously demonstrated that human defensin peptide human α-defensin-5 (HD-5) can block the release of IL-1β, but not tumor necrosis factor-α (TNF-α), from lipopolysaccharide (LPS)-activated human monocytes stimulated with Adenosine triphosphate (ATP)[9]. Here, using HD-5 as a molecular tool, we explored whether IL-1β release is an indivisible process from caspase-1 activation/pro-IL-1β processing in human monocytes.

MATERIALS AND METHODS

Human HD-5

Synthetic human defensin HD-5 was prepared by t-Boc solid-phase synthesis as described previously[9]. All peptides were folded and purified to homogeneity by reversed-phase-high-performance liquid chromatography and their molecular weights verified by electrospray ionization mass spectrometry.

Peripheral blood monocyte isolation

Peripheral blood monocytes (PBMC) from healthy adult donors were prepared as described previously[9]. Briefly, blood was diluted with RPMI 1640, overlaid on lymphocyte separation medium (Mediatech), and centrifuged at 400 × *g* for 35 min. The mononuclear cell layer was washed with PBS and centrifuged at 250 × *g* for 10 min. The cells were then resuspended in 25 mL of PBS plus citrate solution and overlaid on Percoll (GE Healthcare) prediluted 9/1 with 1.5 mol/L NaCl. After a 35-min centrifugation at 400 × *g*, the PBMC were washed with PBS again. Cells were counted using trypan blue exclusion and cells were resuspended in monocyte medium [RPMI 1640, 5%FBS, 20 mmol/L HEPES (pH 7.3), 1%streptomycin/penicillin] and incubated at 37 ℃ for 2 h to allow for adherence, after which medium supernatants were discarded. Attached cells were rinsed twice with monocyte medium and used immediately or incubated in monocyte medium overnight at 37 ℃ in a 5%CO_2 environment.

IL-1β posttranslational processing and release assay

Fresh or overnight cultured PBMCs were treated with 20 ng/mL LPS for 2 h at 37 ℃. In some experiments, the media was removed then replaced with RPMI (without Met, Cys, or Glu), + 1% FBS + 25 mmol/L HEPES pH 7.4 + 300 mg/L Glutamine + 83 μCi/mL ^{35}S-Met/Cys and incubated at 37 ℃ for 1 h. Cells were then rinsed and media was replaced with RPMI (+ Glu). Adenosine triphosphate (ATP, 1 mmol/L), HD-5 (50 μg/mL), and/or nigericin (Nig, 30 μmol/L) was added to culture media and incubated at 37 ℃ for 1.5 h. The supernatants were collected, 1%Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, United States) added, then spun down. Cells were lysed in 500 μL lysis buffer (25 nmol/L HEPES pH 7.4 + 150 mmol/L NaCl + 0.1%Triton X-100 + Protease Inhibitor cocktail) then spun down. Cell lysates and supernatants were subject to IL-1β immunoprecipitation and western blot analysis of caspase-1 as described previously[9]. Results shown are a representative from three independent experiments.

Statistical analysis

All experiments were repeated 3 times to ensure reproducibility. Image J software (NIH) was used to quantitate protein bands. Proteins bands between two different treatment groups were considered statistically significant when $P < 0.05$, by *t*-test analysis.

RESULTS

HD-5 blocks the release of IL-1β from both freshly isolated and overnight cultured monocytes

Our previous studies have shown that HD-5 can block the release of pro-IL-1β and mature IL-1β from ATP stimulated human monocytes that were cultured overnight prior to LPS exposure[9]. Since culturing monocytes *in vitro* can lead to further differentiation into macrophage-like cells[10,11], we determined whether the subcultured monocytes behaved similarly to freshly isolated cells. As shown in Figure 1 (upper panel) the extracellular release

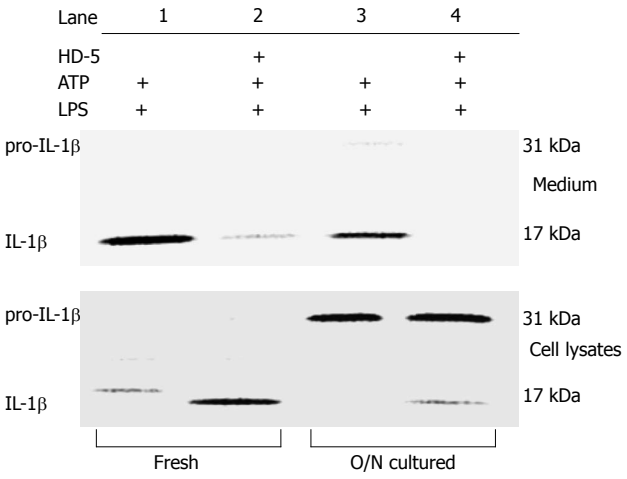

Figure 1 Human α-defensin 5 blocks the release from but not the processing of interleukin-1β in fresh and overnight cultured human monocytes. Freshly isolated or overnight cultured human monocytes were primed with lipopolysaccharide (LPS) (20 ng/mL) for 2 h before being labeled with 35S-methionine/cysteine for 1 h and then washed and treated with Adenosine triphosphate (ATP, 1 mmol/L) and/or human α-defensin 5 (HD-5) (50 μg/mL) for another 1.5 h. Media and cell-associated fractions were harvested separately. Interleukin-1β (IL-1β) was recovered from each by immunoprecipitation with anti-hIL-1β antibodies (1:1000) that recognize both the pro-IL-1β (31 kDa) and mature IL-1β (17 kDa) proteins. The resulting immunoprecipitates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography. Images are representative from three independent experiments.

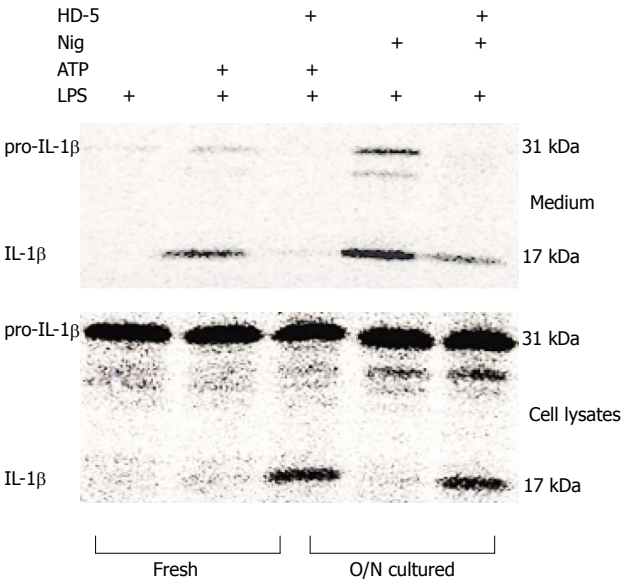

Figure 2 Human α-defensin 5 blocks interleukin-1β release from monocytes stimulated with Adenosine triphosphate or nigericin. Overnight-cultured, lipopolysaccharide (LPS)-activated, 35S-Met/cys-labeled human monocytes were treated with Adenosine triphosphate (1 mmol/L) or nigericin (Nig) (30 μmol/L) in the presence or absence of human α-defensin 5 (HD-5) (50 μg/mL) for 1.5 h. Media and cell-associated fractions were harvested separately. Interleukin-1β (IL-1β) was recovered from each by immunoprecipitation and resulting immunoprecipitates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography. Images are representative from three independent experiments.

of mature IL-1β from both freshly isolated and overnight cultured monocytes was blocked by HD-5. Interestingly, we also observed that freshly isolated monocytes had enhanced intracellular processing of pro-IL-1β to IL-1β (Figure 1, lower panel, lanes 1-2 *vs* 3-4) suggesting that increased time in culture can decrease the ability of monocytes to process IL-1β. Most importantly, it was found that the intracellular processing of pro-IL-1β to mature IL-1β was not inhibited by HD-5 as evidenced by the presence of intracellular mature IL-1β in cell lysates from monocytes treated with HD-5 (Figure 1, lower panel).

HD-5 treatment significantly blocks IL-1β release from monocytes stimulated with Nig

To determine whether the ability of HD-5 to block IL-1β release in overnight-cultured, LPS-activated monocytes was specific to ATP stimulation, we treated LPS-activated monocytes with ATP or Nig. Similar to ATP, Nig is a microbial toxin that acts as an inflammasome inducer, leading to caspase-1 maturation and IL-1β processing and release[12,13]. We found that, similar to its effect on ATP-mediated IL-1β release, HD-5 treatment was also able to significantly block IL-1β release from monocytes stimulated with Nig (Figure 2, upper panel). Furthermore, HD-5 did not block Nig-induced intracellular processing of pro-IL-1β to mature IL-1β as indicated by the presence of mature IL-1β in the cell lysate (Figure 2, lower panel).

ATP and Nig-mediated caspase-1 activation and extracellular release from LPS-activated monocytes is not affected by HD-5

Caspase-1 activation has been implicated in both the processing and release of IL-1β[14,15]. Because our current studies have shown that HD-5 selectively blocked the release but not the processing of pro-IL-1β to mature IL-1β, it is important to know whether HD-5 has any effect on caspase-1 activation and release. As shown in Figure 3, ATP/Nig-mediated caspase-1 activation and extracellular release from LPS-activated monocytes were not affected by HD-5. This observation is consistent with the finding that HD-5 did not block the intracellular processing of pro-IL-1β to IL-1β (Figures 1 and 2).

DISCUSSION

Current research suggests that once caspase-1 becomes active it leads to both the processing and release of IL-1β[16-18]. Although the processing and release of IL-1β are rapid and probably concurrent events, it has been suggested previously that the cleavage of pro-IL-1β and release of mature IL-1β are likely independent of each other[19]. However, this speculation remains hypothetical because, due to the technical limitations, the presence of mature IL-1β inside LPS-primed, ATP-stimulated monocytes have not been documented prior to this report. Here, under certain conditions we have clearly shown that IL-1β release but not its processing from pro-IL-1β by caspase-1 in ATP/Nig-stimulated monocytes can be blocked by HD-5. To our knowledge, this report provides the first direct evidence that the processing of pro-IL-1β to mature IL-1β and extracellular release of mature IL-1β are two divisible events in human monocytes.

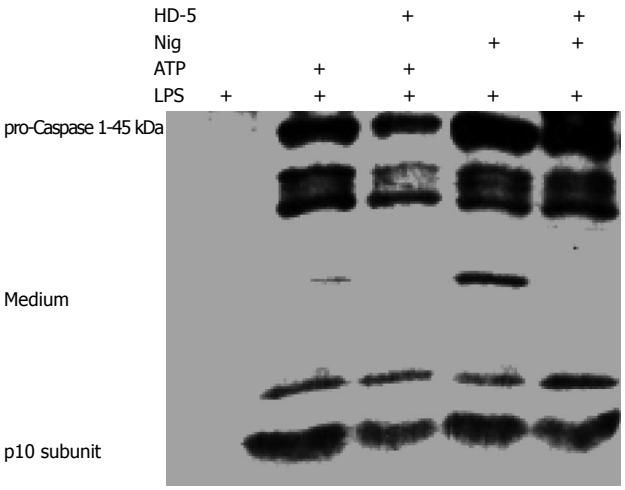

Figure 3 Adenosine triphosphate/nigericin-induced caspase-1 activation and externalization in human monocytes are not blocked by human α-defensin 5. Lipopolysaccharide (LPS)-activated monocytes were stimulated with Adenosine triphosphate (ATP, 1 mmol/L) or nigericin (Nig) (30 μmol/L) in the presence or absence of human α-defensin 5 (HD-5) (50 μg/mL) for 1.5 h. Media were harvested, precipitated, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis with anti-caspase-1 p10 antibodies (1:1000) that recognize both the pro-caspase-1 (45 kDa) and cleaved caspase-1 p10 subunit proteins.

Our results also indicate that the majority of pro-IL-1β is processed intracellularly by activated caspase-1 in freshly isolated monocytes (Figure 1A). This observation is consistent with the report that human blood monocytes release processed IL-1β after a one-time stimulation with toll-like receptor 4 ligands due to the resulting constitutively activated caspase-1[16]. Furthermore, because the release of IL-1β, but not the externalization of caspase-1 was affected by HD-5, our studies suggest that IL-1β seems to be released directly to the extracellular environment without the involvement of caspase-1 inflammasome.

The mechanisms by which pro-IL-1β and mature IL-1β are released from cytokine producing cells have been an intriguing and unsolved question of IL-1β biology for decades. Known activators of caspase-1 and IL-1β release include ATP, adjuvants, and various microbial molecules[20]. Using different experimental systems, studies on ATP-induced IL-1β maturation have posited four different and conflicting models of IL-1β secretion, including secretory lysosome exocytosis[21,22], microvesicle shedding[23-25], direct transport across the plasma membrane[26], and exocytosis of exosome-containing multivesicular bodies[7]. The multiple models of IL-1β secretion reflect the confusion in this area. It is true that although ATP-induced caspase-1 activation is followed by the processing of pro-IL-1β to IL-1β in the cytosol and secretion of IL-1β, other protein release mechanisms, such as externalization of caspase-1, secretory lysosomes and microvesicle shedding, do occur. However, externalization of caspase-1 and other inflammasome components, secretory lysosomes and microvesicle shedding may be cell-type specific processes after ATP stimulation and may not be involved in IL-1β secretion from monocytes. Here, we provide direct evidence that IL-1β secretion is a separable process from inflammasome activation/caspase-1 externalization and IL-1β processing in human monocytes. Inflammasome activation and caspase-1 activity are required but not sufficient for the release of IL-1β.

Taken together, our studies clearly demonstrated that intracellular processing of pro-IL-1β to mature IL-1β and the externalization of mature IL-1β are divisible events in human monocytes. Although the molecular mechanisms by which HD-5 blocks the release of mature IL-1β has yet to be revealed in future studies, a better understanding of IL-1β processing and release may lead to the discovery of novel molecular targets for IL-1β blockade and the development of new therapeutic approaches to treat life-threatening microbial infections and inflammatory diseases.

COMMENTS

Background

Interleukin-1β (IL-1β) is secreted by monocytes and macrophages and is an important acute response factor of host defense against microbial infections. IL-1β is synthesized as a biologically inactive 31 kDa pro-IL-1β polypeptide and must be post-translationally processed by caspase-1 to generate the mature 17 kDa IL-1β that is released into the extracellular space.

Research frontiers

Macrophages deficient in caspase-1 expression have undetectable IL-1β secretion. This suggests that IL-1β release and caspase-1 activation are closely related events. Because most known inhibitors of IL-1β production block caspase-1 activation, previous studies are not able to determine whether caspase-1 activation/pro-IL-1β processing and IL-1β release are separate or linked processes. This report shows that processing of pro-IL-1β by caspase-1 and the release of mature IL-1β from human monocytes are distinct and separable events.

Innovations and breakthroughs

Recent reports suggest IL-1β secretion involves the formation of the inflammasome, leading to the cleavage and activation of caspase-1, which in turn proteolytically processes pro-IL-1β. Biologically active IL-1β is subsequently secreted by the cell. Here authors propose that IL-1β secretion involves a more complex regulatory mechanism.

Applications

Standard therapy for patients with autoimmune diseases or lymphomas involves blocking IL-1β activity. By knowing how IL-1β is processed and released, this study may lead to the development of novel therapeutics that can block IL-1β release and prevent or enhance treatment for these debilitating pro-inflammatory disorders.

Terminology

The inflammasome is a multi-protein complex responsible for the activation of caspase-1, an enzyme that cleaves and activates downsteam targets such as IL-1β. IL-1β is a pro-inflammatory cytokine secreted by immune cells to aid in the defense of microbial infection. Human α-defensin 5 (HD-5) is an anti-microbial peptide that normally functions by binding to the microbial cell membrane to form a lethal pore.

Peer review

The authors examined the ability of HD-5 to block the release, but not the activation, of IL-1β from Adenosine triphosphate and nigericin stimulation human monocytes. It revealed that while HD-5 can block IL-1β release, caspase-1 activation is not affected. The results are interesting and may represent a new molecular mechanism in IL-1β secretion.

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P- Reviewers Holan V, Cairo G
S- Editor Gou SX **L- Editor** A **E- Editor** Lu YJ

ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2013 August 26; 4(3): 35-78

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WJBC

World Journal of Biological Chemistry

Contents

Quarterly Volume 4 Number 3 August 26, 2013

Contents

World Journal of Biological Chemistry
Volume 4 Number 3 August 26, 2013

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World Journal of Biological Chemistry is now indexed in PubMed Central, PubMed, Digital Object Identifier, and Directory of Open Access Journals.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Quarterly

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Telephone: +86-10-85381891
Fax: +86-10-85381893
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PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
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Telephone: +852-6555-7188
Fax: +852-3177-9906
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http://www.wjgnet.com

PUBLICATION DATE
August 26, 2013

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1949-8454/g_info_20100316155305.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
wjbc@wjgnet.com
doi:10.4331/wjbc.v4.i3.35

World J Biol Chem 2013 August 26; 4(3): 35-63
ISSN 1949-8454 (online)

REVIEW

Molecular recognition by gold, silver and copper nanoparticles

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Received: May 2, 2013 Revised: June 11, 2013
Accepted: June 18, 2013
Published online: August 26, 2013

Abstract

The intrinsic physical properties of the noble metal nanoparticles, which are highly sensitive to the nature of their local molecular environment, make such systems ideal for the detection of molecular recognition events. The current review describes the state of the art concerning molecular recognition of Noble metal nanoparticles. In the first part the preparation of such nanoparticles is discussed along with methods of capping and stabilization. A brief discussion of the three common methods of functionalization: Electrostatic adsorption; Chemisorption; Affinity-based coordination is given. In the second section a discussion of the optical and electrical properties of nanoparticles is given to aid the reader in understanding the use of such properties in molecular recognition. In the main section the various types of capping agents for molecular recognition; nucleic acid coatings, protein coatings and molecules from the family of supramolecular chemistry are described along with their numerous applications. Emphasis for the nucleic acids is on complementary oligonucleotide and aptamer recognition. For the proteins the recognition properties of antibodies form the core of the section. With respect to the supramolecular systems the cyclodextrins, calix[n]arenes, dendrimers, crown ethers and the cucurbitales are treated in depth. Finally a short section deals with the possible toxicity of the nanoparticles, a concern in public health.

Key words: Hybrid nanoparticles; Gold; Silver; Copper; Metal; Molecular recognition; DNA; Protein; Supramolecular assembly; Toxicity

Core tip: The article is an in-depth review of the state of the art of molecular recognition processes involving hybrid nanoparticles and bio-molecular substrates. We describe the methods of preparation and physical characterization. The capping by proteins, DNA, peptides and supramolecular assemblies, including cyclodextrins, calix[n]arenes, cucurbitales, dendrimers and crown ethers is then discussed. There is a large analysis of the interactions of these systems with various substrates, such as complimentary oligo-nucleotides, antibodies, active pharmaceutical ingredients and polluants. Finally we discuss the problem of possible toxicity.

Tauran Y, Brioude A, Coleman AW, Rhimi M, Kim B. Molecular recognition by gold, silver and copper nanoparticles. *World J Biol Chem* 2013; 4(3): 35-63 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i3/35.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i3.35

INTRODUCTION

Nanoparticle (NP) science and technology is considered to be a quintessimal aspect of 20th and 21st century

science and research, however the use of Noble metal nanoparticles dates back to at least the Roman epoch[1]. At that time colloidal suspensions of gold were used to stain glass. Somewhat later, in ninth century Mesopotamia copper and silver nanoparticles were used to introduce a metallic luster into pottery glazes[2]. In parallel, in India noble metal nanoparticles were first applied in medicine. During the sixteenth and seventeenth centuries Cassius and Kunckel refined the process of glass staining although still without a fundamental understanding of the subject[3]. Similarly, Herschel developed a photographic process using colloidal gold[4]. It was in 1857 that Faraday first characterized the optical properties of nanoparticles[5]. Subsequent work has shown that the differing colours observed for various forms of noble metal nanoparticles are related to the particle size[6]. With the understanding of plasmon physics, a clear understanding of the behavior of nanoparticles has emerged[7].

In fact, nanoparticles are based on small well defined aggregates of the Noble metals in the zero valent state. The preparation of Noble metal nanoparticles is generally based on a wet chemical reduction of a suitable metal salt in the presence of a capping or stabilizing agent to prevent both aggregation and oxidation away from the reduced state[8]. The size and more importantly the shape of the nanoparticles can be controlled by the reducing agent, the capping agent and the reaction conditions used in the preparation[9]. While spherical forms are most commonly prepared, rod-like shapes, cubes, hexagonal and even hollow forms are known[10].

In this review we will concentrate on how the introduction of various capping agents allows the introduction of molecular recognition properties to the surface of the noble metal nanoparticles. The choice of the capping agent has opened up applications in biomedical science[11], antibacterial systems[12], drug carriers[13] and as sensing elements[14]. The last application, sensing *via* molecular recognition is greatly facilitated by the sensitivity of the wavelength and intensity of the Plasmon Resonance peak to the nature of the local environment around the nanoparticles[15] and also to the aggregation state of the colloidal system[16]. In a final section we will deal with some of the health concerns related to the use of such nanoparticles[17].

PREPARATION AND MODIFICATION OF NANOPARTICLES

Noble metal nanoparticle preparation

Numerous techniques have been developed to synthesize Noble metal nanoparticles, including both chemical methods (*e.g.*, chemical reduction, photochemical reduction, co-precipitation, thermal decomposition, hydrolysis, *etc.*) and physical methods (*e.g.,* vapor deposition, laser ablation, grinding, *etc.*) The ultimate goal is to obtain nanoparticles with a high level of homogeneity and provide fine control over size, shape and surface properties[18].

Table 1 presents the different strategies employed in the literature for preparing and functionalizing metallic nanoparticles.

Nanoparticle functionalization methods

Molecular functionalization on inorganic supports has been made through a variety of techniques that includes physical adsorption, electrostatic binding, specific recognition, and covalent coupling. Recently, these immobilization techniques have been applied to bring together biomolecules and nanoparticles. Willener has reviewed[19] these methods of functionalization and has identified three types: (1) Electrostatic adsorption; (2) Chemisorption; and (3) Affinity-based methods.

The electrostatic adsorption method involves the adsorption of positively charged molecules on nanoparticles that are stabilized by anionic ligands such as carboxylic acid derivatives (citrate, ascorbate). Protein and in particular, antibodies have been used in this way, to functionalize nanoparticles since the work of Faulk and Taylor in 1971[20].

Chemisorption involves capping nanoparticles using the affinity of Noble metals for thiol-containing molecules or by covalent binding through bifunctional linkers. Nucleic acids can be prepared with pendant thiol groups using solid-phase synthesis, thus facilitating their attachment to the metal nanoparticle[21]. Otherwise, an anchor group can be used for covalent binding through bifunctional linkers. Such functionalization can be divided into a two-step process: in 1, the activation step, a chemical anchor layer is formed on the nanoparticle surface to provide active functional groups to which biological molecules (*i.e.*, antibodies) can be covalently attached; and in 2, the functionalization step, biomolecules are covalently linked to the anchor layer to yield systems for molecular recognition[22]. The affinity based method is defined as the functionalization of nanoparticles with groups that provide affinity sites for the binding of biomolecules, and has been used for the specific attachment of proteins and oligonucleotides. For example, streptavidin-functionalized gold nanoparticles have been used for the affinity binding of biotinylated proteins (*e.g.,* immunoglobulins and serum albumins) or biotinylated oligonucleotides[23]. Doria summarized the advantages and disadvantages of the three methods: the advantage of electrostatic adsorption is the ease of usage while chemisorption and affinity-based functionalisations are robust and allow an orientation of the capped molecules. The disadvantages of electrostatic adsorption include sensitivity to the external environment (pH, ionic strength) and the restricted choice of charged molecules for capping. Also, chemisorption and affinity-based functionalization usually require the modification of the capped molecules[24].

PROPERTIES

Optical properties

The optical attributes of metal nanoparticles, as reflected in their bright intense colors, are due to their unique interaction with light. In the presence of the oscillating

Table 1 Summary of various strategies for functionalizing and preparing different metal nanoparticles

Metal	Molecule	Type of conjugation	Nanoparticle preparation	Ref
Gold	Oligonucleotide	Thiol labelled oligo	Citrate reduction	[35,41]
Gold	Aptamer	Thiol labelled oligo	Citrate reduction	[54,56]
Gold	Antisera	Electrostatic attraction	Reduction	[20]
Gold	Antibody : anti-epidermal growth factor receptor	Electrostatic attraction	Citrate reduction	[89]
Gold	Monoclonal antibody LCC (ALT04)	Electrostatic attraction	Citrate reduction	[204]
Gold	Antibody: antigoat, antimouse, anti-sheep	Covalent bond (protein/MUA SAM)	Seed-mediated growth	[91,22]
Gold	Avidin	Succinimyl labelled avidin	Frens-Turkevich method	[71]
Gold	Peptide	Colavelent bonds (peptide-NH2/ COOH-PEG-NP)	seed-mediated growth and coated with tlyolated-PEG-COOH	[75]
Gold	Cyclodextrine	Thiol labelled cyclodextrin	$NaBH_4$ reduction	[119,121]
Gold	Cyclodextrine	Hydrophobic	Laser-induced ablation	[205]
Gold	Calix[n]arene	Thiol labelled calix[n]arene	$NaBH_4$ reduction	[136]
Gold	Calix[n]arene	Thiol labelled calix[n]arene	Citrate reduction	[135]
Gold	Calix[n]arene	Chemisorbtion (sulphonate/gold)	$NaBH_4$ reduction	[138]
Gold	Dendron	Thiol labelled dendron	$NaBH_4$ reduction	[160]
Gold	Dendron	Thiol labelled dendron	Thiol-Ligand Substitution	[161]
Gold	Dendrimer	Electrostatic attraction	Acetic Acid reduction	[158]
Gold	Crown Ether	Thiol labelled crown ether	$NaBH_4$ reduction	[206]
Gold	Crown Ether	Thiol labelled crown ether	Citrate reduction	[178]
Silver	Oligonucleotide	Thiol labelled oligo	$NaBH_4$ reduction	[39]
Silver	Oligonucleotide	Physical adsorption	Photo-Induced	[48]
Silver	Aptamer	Thiol labelled oligo	$NaBH_4$ reduction	[159]
Silver	Antibody: IgG	Electrostatic attraction	Citrate reduction	[207]
Silver	Antibody: anti-ngn1	Electrostatic attraction	Citrate reduction and coated with DL-mercaptosuccinic acid (MSA)	[101]
Silver	Norvancomycin	Covalent bond (peptide-NH2/ COOH-MA-NP)	$NaBH_4$ reduction stabilized with Mercaptoacetic Acid (MA)	[82]
Silver	Glutheraldehyde	Electrostatic attraction	$NaBH_4$ reduction	[104]
Silver	Peptide	Electrostatic attraction	Ascorbate sodium reduction	[76]
Silver	Peptide	Electrostatic attraction	trisodium citrate and hydroxylamine hydrochloride reduction	[77]
Silver	Peptide	Electrostatic attraction	NaOH added to silver nitrate and peptid	
Silver	Cyclodextrin	Host-guest by Electrostatic interaction	$NaBH_4$ reduction stabilized with Cetyl Trimethyl Ammonium (CTA)	[208]
Silver	Cyclodextrin	Thiol labelled cyclodextrin	$NaBH_4$ reduction	[127]
Silver	Cyclodextrin	Thiol labelled cyclodextrin	Citrate reduction	[126]
Silver	Cyclodextrin	Electrostatic attraction	$NaBH_4$ reduction	[124]
Silver	Calix[n]arene	Chemisorbtion (sulphonate/silver)	$NaBH_4$ reduction	[145,147]
Silver	Calix[n]arene	Electrostatic attraction	Hydrogen gaz reduction	[143]
Silver	Calix[n]arene	Electrostatic attraction	Photo-chemical reduction	[144]
Silver	Dendrimer	Electrostatic attraction	UV reduction	[164,168]
Silver	Dendrimer	Electrostatic attraction	$NaBH_4$ reduction	[166]
Silver	Crown Ether	Thiol labelled crown ether	$NaBH_4$ reduction	[177]
Silver	Cucurbit[n]uril	Chemisorbtion	$NaBH_4$ reduction	[179]
Silver	Cucurbit[n]uril	Chemisorbtion	NaOH induced	[180]
Copper	Oligonucleotide	Electrostatic attraction	Chemical reduction (ascorbic acid)	[53]
Copper	Antibody	Electrostatic attraction	Pyrometallurigically by heating copper metal (Sigma, China)	[109]
Copper	Peptide	Electrostatic attraction	NADH reduction by fungus F. oxysporum	[84]
Copper	Peptide/latex	Electrostatic attraction	Ascorbate sodium reduction	[83]
Copper	Cyclodextrin	Electrostatic attraction	ultrasound irradiation	[128]
Copper	Cyclodextrin	Electrostatic attraction	calcination	[129]
Copper	Calix[n]arene	Chemisorbtion (sulphonate/copper)	hydrazine reduction	[151]
Copper	Dendrimer	Electrostatic attraction	Ascorbic acid reduction	[171]
Copper	Dendrimer	Electrostatic attraction	Electrochemical reduction	[170]

electromagnetic field of light, the free electrons of the metal nanoparticle undergo a collective coherent oscillation (Figure 1). This motion is resonant at a particular light frequency and is termed the localized surface plasmon resonance (LSPR) oscillation[25]. The surface plasmon oscillation either decays by radiating its energy, resulting in light scattering, or decays non-radiatively as a result of conversion of absorbed light to heat. The electric field intensity and the scattering and absorption characteristics of the nanoparticles are all strongly enhanced

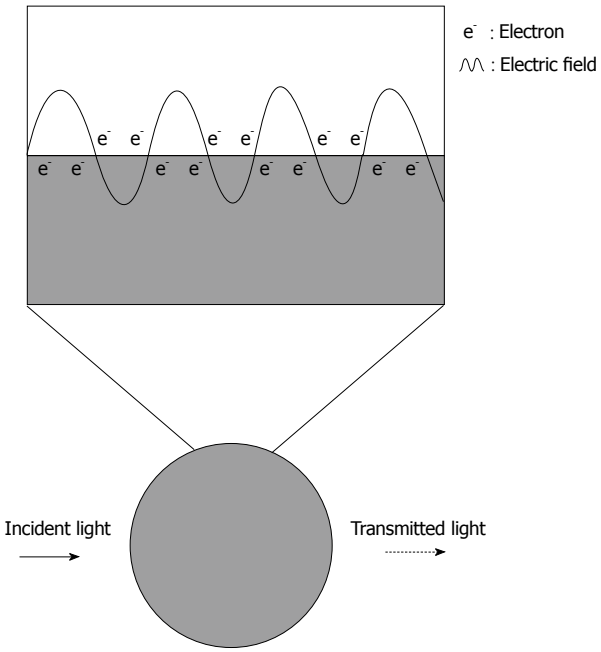


Figure 1 Scheme representing the plasmonic effect induced by white light on the absorbance of a silver nanoparticle. The plasmon is represented above by the oscillation of an electron cloud along the surface of the nanoparticle. The silver nanoparticle absorbs light at 390 nm giving a dotted line.

at the LSPR frequency, which for gold, silver and copper lies in the visible region[26].

The plasmonic resonance of metallic nanoparticles will depend on several parameters: (1) the size of the nanoparticles; (2) the geometry of the nanoparticles (spherical, triangle, rods, *etc.*); (3) the physical properties of the medium in which the nanoparticles are dispersed (air, liquid, solid); and (4) the nature of the metallic nanoparticles.

In water, the absorption of spherical nanoparticles, with sizes ranging from 3 nm to 80 nm and composed of copper (Cu), silver (Ag) or gold (Au), lies in the visible range and gives rise to a narrow peak[27], respectively at 400, 520 and 570 nm. The metal plasmon absorption frequency for copper, silver and gold nanoparticles were 500-550 nm, 390-400 nm, 565-570 nm respectively.

Electrical properties

The electrical properties of metal particles which have a size greater than 2 nm diameter, are similar to those of the corresponding bulk metals[28]. Electron transport is not confined to the discreet energy levels of several atoms but appears as a continuum energy level of a bulk metal. Hence, surface charging and electron transport processes in metal nanoparticles may be understood with relatively simple classical physical expressions, as for resistance/capacitor electronic circuit diagrams. In contrast to molecules and semiconductor nanoparticles whose electron transport properties require a quantum mechanical description, metal nanoparticles only require knowledge of their size and the dielectric properties of the surrounding medium to determine their properties[18]. The electronic properties of metallic nanoparticles have been used for many applications, such as electrical sensors using metal nanoparticle as a tag for recognizing a specific target molecule[29], and the development of new electronic chips[30].

COATING AND MOLECULAR RECOGNITION

Nucleic acid coating

After proteins, the nucleic acids are the most studied biomolecules for capping Noble metal nanoparticles. Many reviews regarding gold nanoparticles, can be found in the literature[31-34]. Such hybrid systems have been extensively investigated since Mirkin, first used oligonucleotides as a capping agent to provide a basis for recognition[35]. Nucleic acid molecules consist of a sequence of nucleotides distinguished by which nucleobase they contain. In DNA, the four bases present are adenine (A), cytosine (C), guanine (G), and thymine (T), whereas RNA contains adenine (A), cytosine (C), guanine (G), and uracil (U). Nucleic acids have the property that two strands will only bind to each other to form a double helix if the two sequences are complementary, with A only binding to T in DNA A binds to U in RNA, and C only to G, linked by hydrogen bonds (Figure 2).

Moreover, the ability of nucleic acids to self assemble extends their molecular recognition properties from complementary sequences to various molecular targets such as small molecules, proteins, and even cells, tissues and organisms[36]. Aptamers (Apt), oligo-nucleic acids engineered to bind a specific ligand, have shown considerable potential to be used as a capping agent for nanoparticles[21].

Oligonucleotides: As noted above, oligonucleotides bind, in a sequence-specific manner, to their respective complementary oligonucleotides, DNA, or RNA to form duplexes or, less often, hybrids of a higher order. This basic property serves as a foundation for the use of oligonucleotides as probes for detecting DNA or RNA. Many applications can be found in biology such as Polymerase Chain Reaction (PCR), DNA microarrays, Southern blots, fluorescent *in situ* hybridization (FISH)[37]. As an example, DNA microarrays use thousands of different oligonucleotides probes in order to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. The fundamental idea behind most microarrays is to exploit complementary base pairing of the oligonucleotide probes to measure the amount of the different types of mRNA molecules in a cell, thus indirectly measuring the expression levels of the genes that are responsible for the synthesis of those particular mRNA molecules[38].

Gold: Oligonucleotide Gold Nanoparticle (OGN) conjugates are powerful tools for the detection of target DNA sequences by the complementary assemblage of double stranded DNA. Practically all the research and applica-

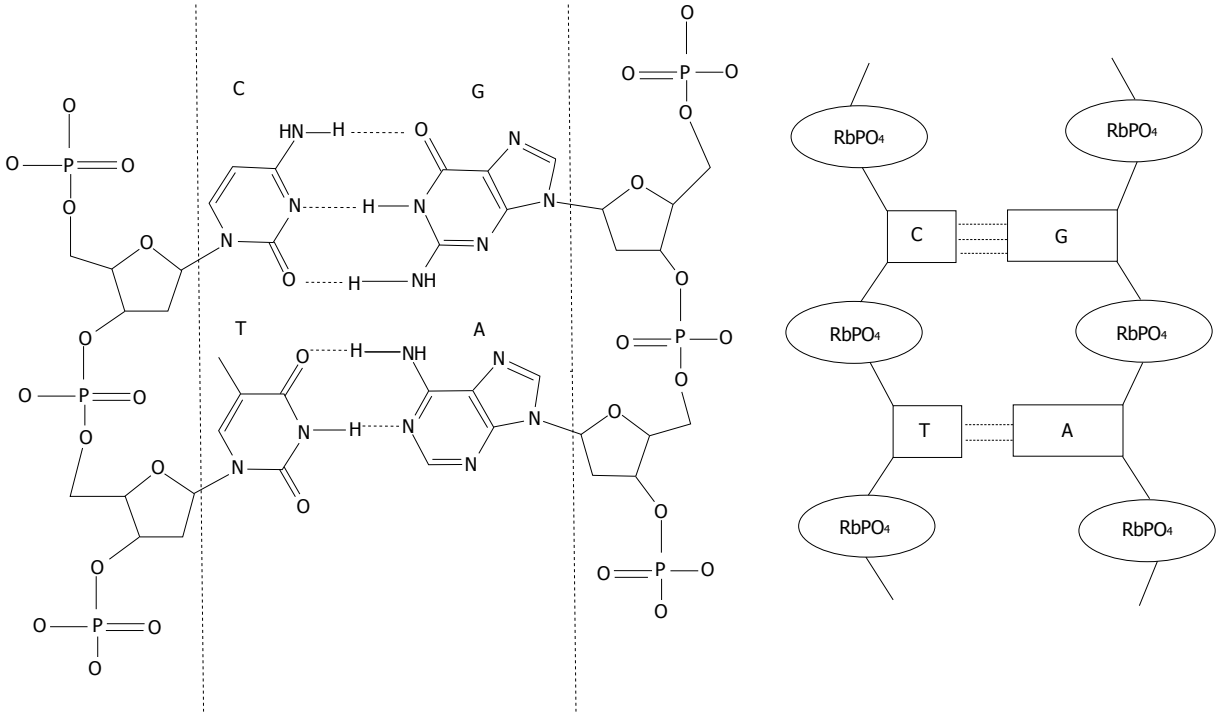


Figure 2 Matching DNA base. Nucleotides structures are represented between the dashed lines. A (adenine) match with T (thymine), C (cytosine) match with G (guanine). Ribose phosphate structures link the nucleotides. On the left, a scheme representing the double stranded DNA based on the sequence nucleotide recognition. $RbPO_4$ is ribophosphate.

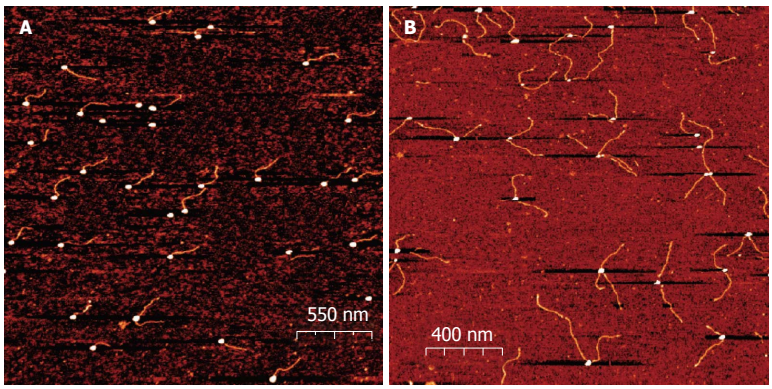


Figure 3 Atomic Force Microscopy images of oligonucleotide Oligonucleotide Gold Nanoparticles conjugates. The conjugates were deposited on a mica surface in 2 mmol/L $MgCl_2$ and scanned in a semi contact mode as described. From Borovok *et al*[46], reproduced with permission from American Chemical Society Publications.

tions of these conjugates have used gold nanoparticles to the exclusion of other Noble metal nanoparticles[39]. Initially, Mirkin demonstrated the colorimetric detection of hybrid gold nanoparticles[35]. Subsequently Mirkin used non-complementary thiolated oligonucleotide probes attached by chemisorption on 13 nm gold nanoparticles[40], addition of DNA containing the complementary sequence for both oligonucleotide probes led to aggregation of the nanoparticles.

Subsequent biological applications led to the development of an oligonucleotide gold nanoparticle set for the detection of mutation of a polynucleotide sequence[41]. With the development of DNA arrays, oligonucleotide capped gold nanoparticles have been shown to be alternative markers to classical fluorophores, bringing very high sensitivity (50 fM of targeted DNA)[42].

Sun *et al*[43] demonstrated the ability to use such a hybrid system for multiple DNA sequence detection by the surface-enhanced raman scattering (SERS) technique. The assembly of nanoparticles provoked by molecule junctions, enhances strongly the Raman scattering[44]. This has been used for detecting and identifying each set of oligonucleotide capped gold nanoparticles which hybridize with the unknown DNA.

The emergence of DNA origami, first described by Rothemund[45], offered new applications of nucleic acid capped gold nanoparticles. In electronic and plasmonic applications, the self assembly properties of DNA can be associated with metallic nanoparticles to construct a variety of metallized and nanostructured shapes. Borokov has developed a method for controlling a specific number of short (25 base) ssDNA molecules[46]. This method allows variation in the size of nanoparticles, the distance between them (by changing the length of a DNA linker), and the number of connections that each particle establishes (Figure 3). Such work opens up horizons for

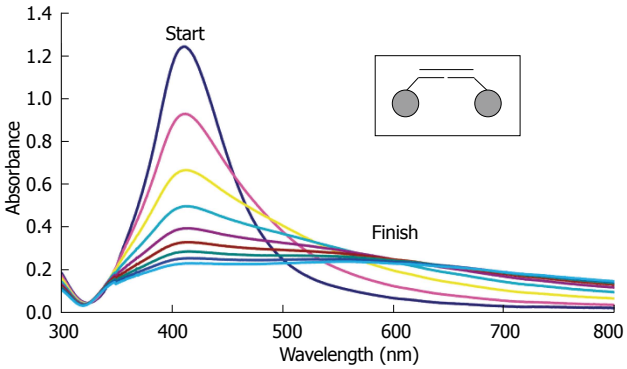


Figure 4 UV-vis spectra taken every 5 min of Oligonucleotide Silver Nanoparticles conjugates (25 pmol/L) hybridizing to a fully complementary target oligonucleotide (2.5 nmol/L). Full spectrum scans were taken every 10 min for 80 min. The inset shows that the conjugates are hybridizing in the "tail-to-tail" juxtaposition. From Thompson *et al*[39], reproduced with permission from American Chemical Society Publications

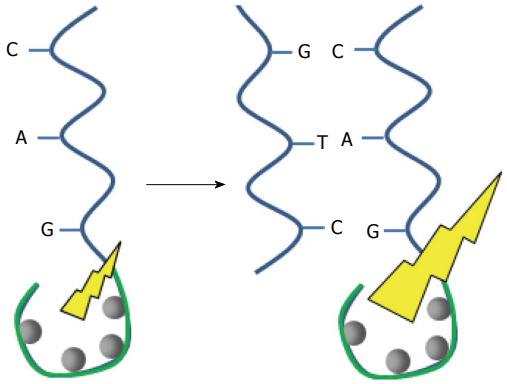


Figure 5 Schematic representation of the DNA sequence detection by fluorescence enhancement of silver nanocluster. A: Adenine; T: Thymine; C: Cytosine; G: Guanine.

nucleic acid capped gold nanoparticles to be used for developing new functional materials.

Silver: In 2008 Thompson reported the synthesis of oligonucleotide silver nanoparticle conjugates and demonstrated their use in a sandwich assay format[39] (Figure 4). These conjugates have practically identical properties to their gold analogues and due to their greater extinction coefficient, absorption analyses can occur at much lower concentrations.

Li *et al*[47] showed that oligonucleotide silver nanoparticle conjugates could be developed for multiplexed DNA electrochemical detection. Although the emergence of DNA chip technology has accelerated this process, it is still a challenge to perform ultrasensitive DNA assays at attomol concentrations. With the use of oligonucleotide silver nanoparticles and a suitable device, it is possible to detect concentrations as low as 5 aMol/L of viral DNA.

Recently Zon *et al*[48] reported the photo-induced nucleation and growth of silver nanoparticles in the presence of DNA oligomers. An organic dye (Cy5) was used as a photosensitizer to initiate nanoparticle growth. Irradiation of the precursor solutions with light at the Cy5 absorption maximum triggered the instantaneous formation of spherical particles with a metallic core of 15 nm in diameter.

The emergence of a new class of so-called Oligonucleotide Capped Silver Nano Clusters (OSNC), consisting of silver nanoparticles from 2 to 10 atoms of silver (2 nm maximum), has led to the observation of novel fluorescence properties, including tunable emission and high photostability. At these sizes, discrete atomic energy levels merge into highly polarizable, continuous, plasmon-supporting bands, thereby leading to very strong absorption and emission[49]. The preparation is very simple, requiring mixing of a suitable salt of silver with the desired oligonucleotide and addition of a reducing agent. Interestingly, the fluorescence properties depend on the sequence of the oligonucleotide. The main applications are sensors for genes, proteins, small molecules, or metal anions. For example, OSNC can be used instead of oligonucleotide probes tagged with a fluorophore to detect a targeted DNA sequence. Their hybridization with a targeted DNA sequence enhances the fluorescence (Figure 5). Chang has developed a system able to detect single point mutations in the gene involved in the hereditary disease tyrosinemia type I [50]. Additionally, these structures promise potential for labeling biomolecules for imaging purposes, as they can be prepared to yield fluorescence in the red or NIR. Antibodies have been conjugated for imaging NIH 3T3 cells[51].

Copper: Because of their ease of back-oxidation, copper systems are still not widely used as nanoparticle cores. However, oligonucleotide capped copper nanoclusters have recently been developed and offer an excellent choice as functional biological probes. Rotaru showed that copper can selectively metallize a double stranded DNA on a oligonucleotide probe, allowing control of the size of the cluster[52]. Moreover, the fluorescence of DNA-hosted Cu nanoclusters is very sensitive to the base type located in the major groove. The advantages of this method over current fluorescence based assays employed for the detection of mismatches in DNA (as Real time quantitative PCR) are a simpler design of probes and the fact that no conjugation of fluorophore on the probes is required[53].

Apt: In comparison to antibodies, Apt possess certain advantages, including their relatively simple and inexpensive synthesis, tolerance to internal labeling, and long storage times without losing their biological activity. The use of Apt as capping agents for metal nanoparticles started in 2004, when Pavlov used such nanoparticles to detect thrombin with a Quartz Crystal Microbalance (QCM)[54]. The resonance of the QCM is disturbed by small mass changes due to film deposition on the surface of the acoustic resonator. Here, the advantage of using metallic particles is to give a weight effect so as to increase the sensitivity of the targeted molecule (Figure 6).

Chang has used Apt gold nanoparticles as biosensors for detecting Platelet-Derivated Growth Factor (PDGF)[55], which is overexpressed in some cancer cells.

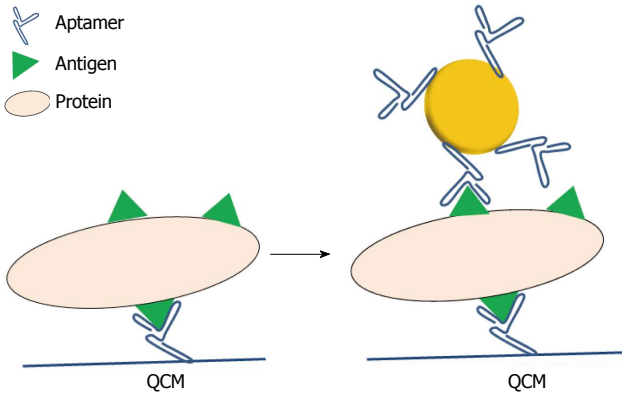


Figure 6 Schematic representation of the detection of proteins on a Quartz Crystal Microbalance using aptamer capped gold nanoparticles.

They proposed an aggregation-based assay using Apt gold nanoparticles to detect PDGF at the nM level. By the same principle, other Apt gold nanoparticles were developed toward adenosine triphosphate and glutathione[56].

The same group later used Apt gold nanoparticles as a contrast agent for detecting cancer cells over-expressing PDGF[57]. Nanoparticles enter the cell where PDGF induces aggregation, and produces a colour (Figure 7). When compared to immunostaining, this approach offers advantages of lower cost and minimum matrix interference. Similarly, Liu used Apt gold nanoparticles for recognizing cancer cells with a strip biosensor[58].

Very recently, one study described the use of silver as a nanoparticle material[59]. In this case silver was chosen because of its excellent optical properties for metal enhanced fluorescence[60]. Based on the results, an aptamer based fluorescent switch has been constructed. In the "OFF" state, without the target molecule, there is a greater spacing distance between the dye and the silver nanoparticle giving comparatively lower fluorescence intensity. However in the "ON" state, in the presence of target molecules, the fluorescence signal is increased due to a shortened distance between the dye and the nanoparticle (Figure 8). This Apt-sensor linearly detects adenosine concentrations from 200 nmol/L to 200 μmol/L with a detection limit of 48 nmol/L.

Protein coatings

The structural versatility, biological importance and biomedical impact of proteins make them one of the most widely studied classes of molecules in bio-recognition. There has long been a major interest in the use of capping metallic nanoparticles with proteins[19,61]. Among proteins with molecular recognition abilities, antibodies, key elements of the immune response, are probably the most widely studied[62,63]. Enzymes have also been studied as capping agents for metallic nanoparticles. Willner *et al*[64] reviewed recent advances in the development of enzyme-metallic nanoparticle conjugates and their specific applications for biosensing and the generation of nanostructure.

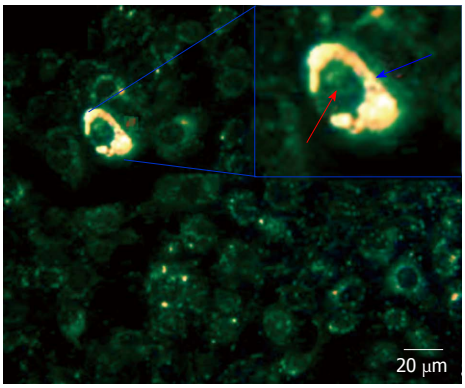


Figure 7 Dark Field Microscopy images of mixtures of cancer MDA-MB-231 and normal 184B5F5/M10 cells at 1:100 ratio, after incubation for 3 h in a medium containing aptamer gold nanoparticles. Gold nanoparticles showing a high reflection are colored in yellow. The lower reflection corresponding to the cells are colored in green. In the inset, a magnification of a cell containing nanoparticles. The distribution of nanoparticles is highlighted by blue arrow corresponding to cytoplasm. Red arrow corresponds to the cell nucleus[57].

Peptides: Peptides which are distinguished from proteins on the basis of size (typically containing fewer than 50 monomer units), and their folding, are well known to be involved in molecular recognition events. Peptide-capped metal nanoparticles combine several advantages. Peptide chemistry is versatile and provides the possibility to utilize functional groups found in the 20 naturally occurring amino acids plus the possibility to introduce non-natural amino acids[65]. Peptides and peptide conjugates are readily commercially available. The preparation of peptide-capped nanoparticles is rapid, simple, and amenable to high-throughput approaches. This allows, in a single step, the production of stable and functional nanoparticles. Peptide-capped gold nanoparticles and, more generally, peptide-capped nanomaterials have immediate applications as bioanalytical sensors and cell imaging, but, perhaps more importantly, they offer an almost unlimited range of possibilities for the design and preparation of the advanced functional nanomaterials of the future[66].

Gold: The use of peptide-capped gold nanoparticles as enzyme mimics, also called nanozymes has been previously reviewed[67,68]. The fundamental idea is to engineer a micro environment, within a self assembled monolayer, that resembles the catalytic site of natural enzyme. Pengo developed a functional artificial protein by grafting a thiol functionalized dodecapeptide onto the surface of gold nanoparticles. This system was able to catalyze the hydrolysis of carboxylate esters. It was found that certain substrates affected the structure of the catalytic site by altering its hydration, demonstrating that the nanozyme can regulate its own activity just as proteins do[69].

Brust used peptide-capped gold nanoparticles as artificial substrates for kinases to develop a colorimetric protocol for the evaluation of kinase activity and inhibition[70]. Sun reported the use of these hybrid particles in a microarray format[71]. The method is based on labeling peptide phosphorylation events on a microarray with

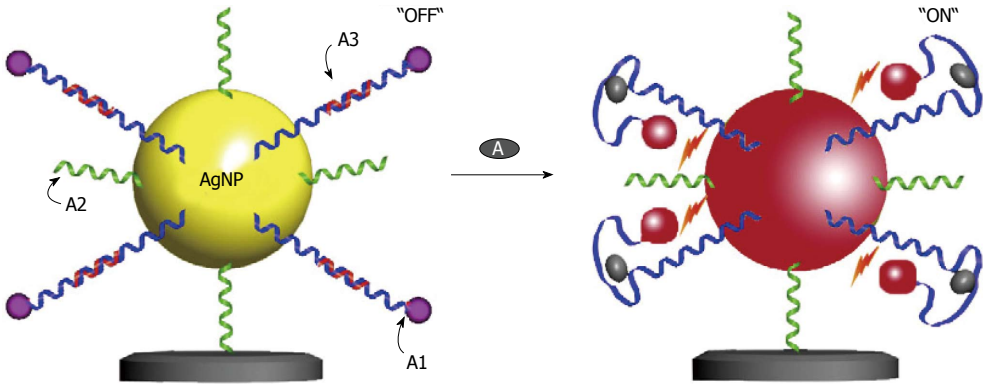


Figure 8 Schematic diagrams of the aptamer-based silver nanoparticles nanosensor showing the "OFF" (a) and "ON" (b) state based on the spacing distance between the Cyanine 3 and the silver nanoparticle surface in the detection of adenosine. From Wang *et al*[59], reproduced with permission from Elsevier.

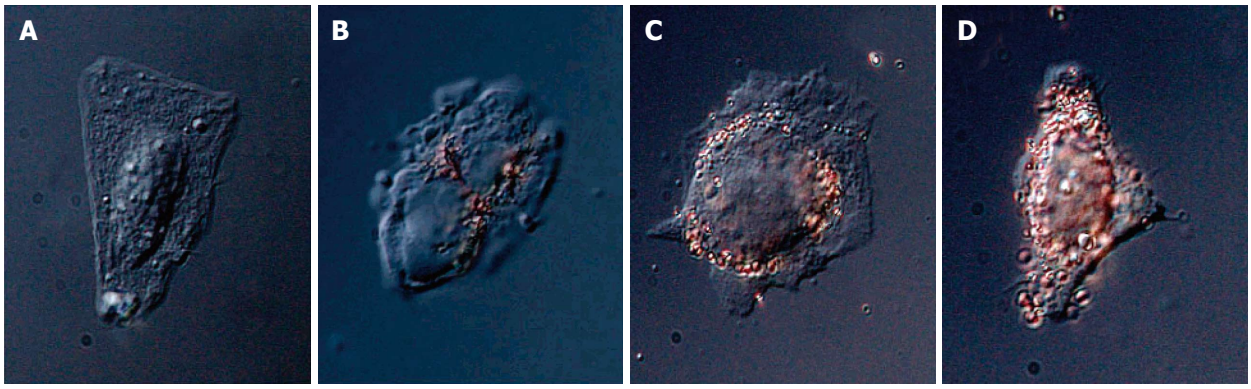


Figure 9 Nanoparticle-peptide complexes incubated with HepG2 cells for 2 h: displaying 4 different sequences in A, B, C and D. From Tkachenko *et al*[74], reproduced with permission from American Chemical Society Publications.

gold nano-particles and using resonance light scattering (RLS) detection[72]. They demonstrated that it is possible to screen kinases with single or multiple inhibitors simultaneously on the same microarray[73].

Targeting transmembrane transport is another field of application in which peptide-capped gold nanoparticles have been used. Franzen reported a method for assessing the efficiency of various combinations of targeting peptides using nanoparticle complexes for nuclear targeting. VEC-DIC combination microscopy permits observation of the localization of the peptide-capped gold nanoparticles according to the peptide sequence[74] (Figure 9). Recently El Sayed investigated the quantitative tumor uptake of a class of elongated gold nanoparticles (nanorods) that were covalently conjugated to tumor-targeting peptides[75]. The results suggest that for photothermal cancer therapy, the preferred route of gold nanorod administration is intratumoral injection. With direct tumor injection, the peptide-capped gold nanoparticles were mainly found in the tumor cells while peptide-capped gold nanoparticles injected with intravenous injection are mainly localized around blood vessels, in the tumor stromal matrix.

Silver: To date, few articles describe the use of peptide capped silver nanoparticle for colorimetric sensing. Most studies focus on the nature of the peptide/silver interaction and the effect of the peptide on the formation of the silver nanoparticles[76,77]. Recently, Cui has demonstrated that at basic pH the peptide secondary structure was modified, and could affect the size of the silver nanoparticles[78].

Qu reported a homogenous assay for colorimetric and quantitative detection of a cancer marker and the promising antitumor target, cyclin A2, using the aggregation of unmodified gold nanoparticles and/or silver nanoparticles[79]. They used the difference in coagulating ability of a cationic peptide probe (P1) and its binding form toward unmodified nanoparticles, in order to detect cyclin A2. In the absence of cyclin A2, P1 aggregates particles immediately, whereas cyclin A2 binding prevents the interaction of P1 with the surface, significantly reducing the aggregation. The extent of aggregation is dependent on the concentration of the target protein cyclin A2 and the difference in color can readily be distinguished by spectrometer (Figure 10).

Generally, peptide capped silver nanoparticles are used for their anti-microbial activity. Taglietti studied the mechanism of action of glutathione (GSH) capped nanoparticles. GSH peptide displays a thiol function, capable of being anchored to silver surfaces, and three pH-dependent, charged functional groups (carboxylates and amines), that promote water solubility and interactions with complex biostructures[80]. GSH capped nanoparticles have shown a bacteriocidal effect related to their penetra-

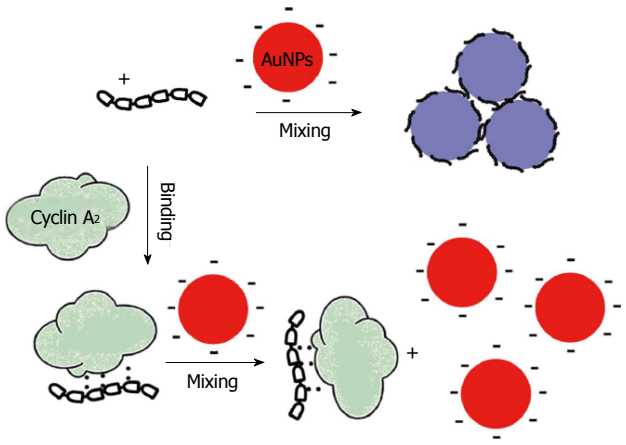


Figure 10 Schematic illustration of colorimetric detection of cyclin A2 based on noncross linking aggregation of unmodified Gold nanoparticles induced by preferential adsorption of unbound P1. From Wang *et al*[79], reproduced with permission from Elsevier.

tion of bacterial cells. They show a stronger bacteriocidal effect on *Escherichia coli* (*E. coli*) (Gram negative strain) than on *Staphylococcus aureus* (gram positive strain). Moreover GSH capped Ag nanoparticles showed lower antibacterial activity when grafted onto functionalized glass surfaces, which prevent the nanoparticle from penetrating the bacterial cell[81]. Wei *et al*[82] investigated another mechanism of the antibacterial effect using the peptide norvancomycin, a treatment of choice for antibiotic resistant bacteria. The silver nanoparticles decrease the stability of the lipopolysaccharide (LPS) present in the outer membrane of gram negative strains. Using the molecular recognition ability of norvancomycin, the hybrid nanoparticles bind to the peptidoglycan inner membrane and improve the destruction of the bacteria by increasing access of norvancomycin.

Copper: Peptide capped copper nanoparticles have received less interest. Recently, Thakore produced peptide-capped copper nanoparticles of 12-16 nm by chemical reduction[83]. The synthesis was carried out using stem latex of the medicinal plant, *Euphorbia nivulia*. The nanoparticles were stabilized and subsequently capped by peptides and terpenoids present within the latex. The study demonstrated that peptide capped copper nanoparticles are toxic to A549 cells in a dose dependent manner. Cell viability assay (MTT) determined an LD_{50} concentration of 20 μg/mL. The dose dependent cytotoxicity (biocompatible below 1 μg/mL) suggests that these nanoparticles could be used in the future to induce apoptotic destruction of cancer cells. Hossieni reported a promising method for producing such hybrid compounds[84]. The group reported the bio-production of copper sulfide nanoparticles from $CuSO_4$ solution by the reduction of NADH released from the fungus *Fusarium oxysporum*. transmission electron microscopy (TEM) images demonstrated that spherical particles of 2-5 nm, were enclosed in spherical peptide shells of about 20 nm in diameter.

Antibodies: Antibodies constitute one of the most important specific defense mechanisms in vertebrate animals. All of them are bifunctional molecules in a Y-form with two identical domains for antigen recognition (Fab fragment), and two identical domains with effector functions (Fc fragment). The antigen-binding region is highly specific to individual antibodies and large numbers of different antibodies are available[85]. Antibodies act as neutralizers of pathogens or toxins, as well as in the recruitment of immune elements (complement, phagocytosis, antibody dependent cytotoxicity by natural killer cells, *etc*.). In addition they may transport molecules including toxins, drugs, fluorophores, and be used in diagnostic procedures, or in therapy to destroy a specific target. The conjugation of antibodies to nanoparticles generates a versatile product that combines, the small size and their thermal, electrical, or optical characteristics of nanoparticles with the abilities of antibodies for specific and selective recognition[61,86].

Gold: So far, antibody capped gold particles are the most popular hybrid systems for the exploitation of the molecular recognition properties of the capping agent. In 1971, Faulk reported their use as a specific marker for Salmonella surface antigens using TEM because of their high electron-dense metal density[20]. Later Horisberger demonstrated their use for Scanning Electron Microscopy[87]. Subsequently, Sokolov showed that when 12 nm gold nanoparticles were conjugated to anti-epidermal growth factor receptor (anti-EGFR) antibodies, they specifically bound to EGFR proteins overexpressed on the surfaces of cervical cancer cells[88]. Illumination of nanoparticle-labeled cells with laser light lit up the gold nanoparticles, and thus, the associated cancer cells. Later, El-Sayed used simple dark field optical microscopy to detect gold nanoparticle-labeled cancer cells. Anti-EGFR antibody conjugated gold nanoparticles were incubated with a nonmalignant epithelial cell line (HaCaT) and two malignant oral epithelial cell lines. The hybrid nanoparticles bound to the surface of the cancer type cells with 600% greater affinity than to the noncancerous cells[88].

Within the last decade, there has been substantial interest in antibody capped gold based contrast agents for *in vivo* X-ray imaging[89,90]. They are a promising candidate for next generation X-ray contrast materials, and their use in combined radiotherapy is under evaluation[91].

One of the most promising aspects of gold nanoparticle use in medicine, is targeted drug delivery. The most popular objects for targeted delivery are antitumor drugs[33]. Combination with antibody capping has been demonstrated to increase the intracellular uptake of gold nanoparticle carriers as compared to non-functionalized conjugates[92]. Chan investigated the cellular uptake of antibody-functionalized gold nanoparticles of different sizes, 2-100 nm[93]. It was found that nanoparticles with diameter of 40-50 nm enter cells more efficiently than either smaller and larger sized gold nanoparticles. At this size the antibody-capped gold nanoparticles maximize interactions with cell surface receptors and thus enter *via* receptor-mediated endocytosis (Figure 11)[94].

Another promising therapeutic application of antibody

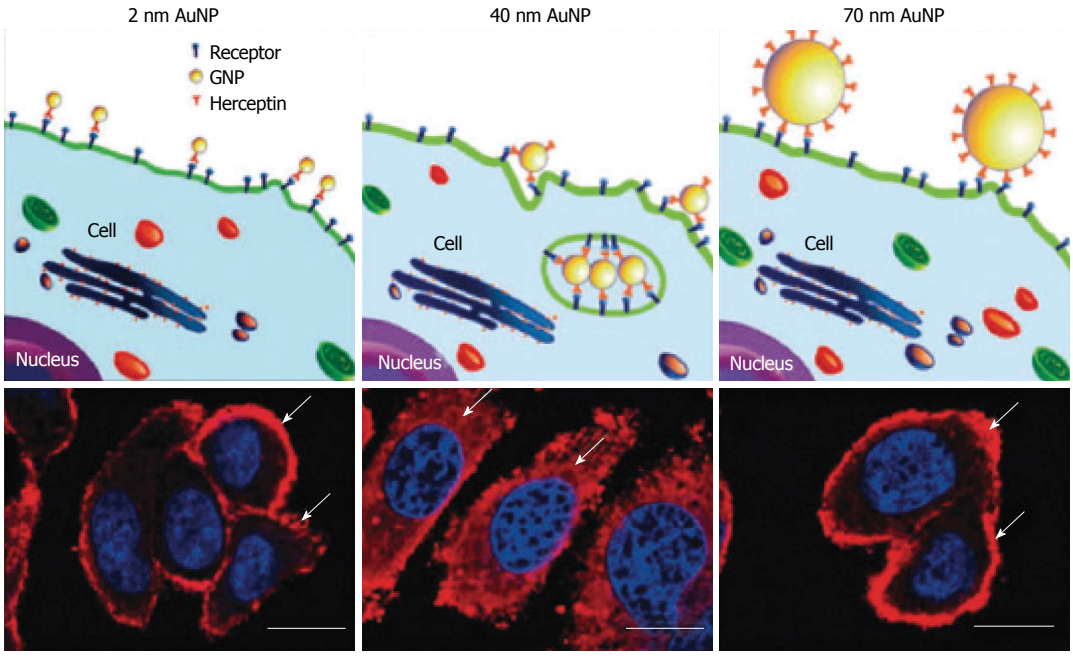


Figure 11 Illustration demonstrating binding of gold nanoparticles (G2, G40, G70 for 2, 40, 70 nm diameter) functionalized with Herceptin antibodies, which recognize receptors on the cell surface. Arrows indicate ErbB2 receptors, and the nucleus is counterstained in blue with 4,6-Diamidino-2-phenylindole, scale bar = 10 μm. From Jiang *et al*[94], reproduced with permission from Nature Publishing Group. AuNP: gold nanoparticles.

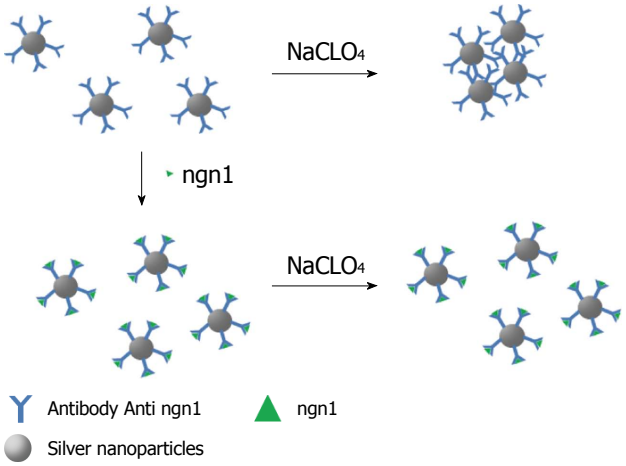


Figure 12 Schematic illustration of ngn1 detection using the anti-ngn1 antibody conjugated silver nanoparticle mediated by $NaClO_4$ salt.

capped gold nanoparticles concerns photothermal damage to cells. Current research is focused on the treatment of cancer and infectious diseases[95]. Gold nanoparticles have an absorption maximum in the visible or near infrared region and become very hot when irradiated at the resonant frequency. If the gold nanoparticles are located inside or at the surface of target cells, these cells will overheat and die. Such work has been reviewed in depth[96,97].

Finally, gold nanoparticle-antibody conjugates have been used for as biosensors for their complementary antigens. A wide variety of such antibody-antigen interactions been reviewed[19,24,33]. As an illustrative example, the aggregation of gold nanoparticles by antigen-antibody interactions in solution was applied to develop immunoassay procedures with optical detection of the association process[98,99]. A laser based double-beam absorption detection system for aggregation immunoassays has been developed. The assay is based on the aggregation of antigen capped gold nanoparticles, in the presence of the corresponding antibodies. The aggregation of the gold nanoparticles resulted in a change in the absorption bands with a detection limit for an antibody of 3×10^{-8} M.

Silver: A range of highly sensitive biosensing methods using antibodies have been developed by exploring different physicochemical properties of the Noble metal nanoparticles, such as LSPR, metal fluorescence enhancement/quenching, SERS, electrochemical activity, *etc*[24]. To date, a number of colorimetric sensors using silver nanoparticles as probes have been developed[100]. In an effort to overcome the lower stability of silver nanoparticles Yuan reported a colorimetric sensing scaffold for neurogenin 1 (ngn1), a peptide expressed in neuronal precursor cells with the function of controlling the differentiation of neurons[101]. The detection procedure is based on an anti-aggregation mechanism, by which ngn1 inhibits the aggregation of the probe in the presence of $NaClO_4$. The anti-ngn1 antibody conjugated silver nanoparticles (AgNP-Ab) is negatively charged, and binding of the negatively charged ngn1 to the probe enhances interparticle electrostatic repulsion. Accompanying the increase of ngn1 concentration, the color of the solution varies from red to yellow, presenting an approach for the detection of ngn1. This assay exhibits a linear response range over nearly two orders of magnitude, from 50 to 800 ng/mL, and a detection limit of 30 ng/mL (Figure 12).

In the area of micro- and nanotechnology-derived biosensors the use of antibody capped silver nanoparticles

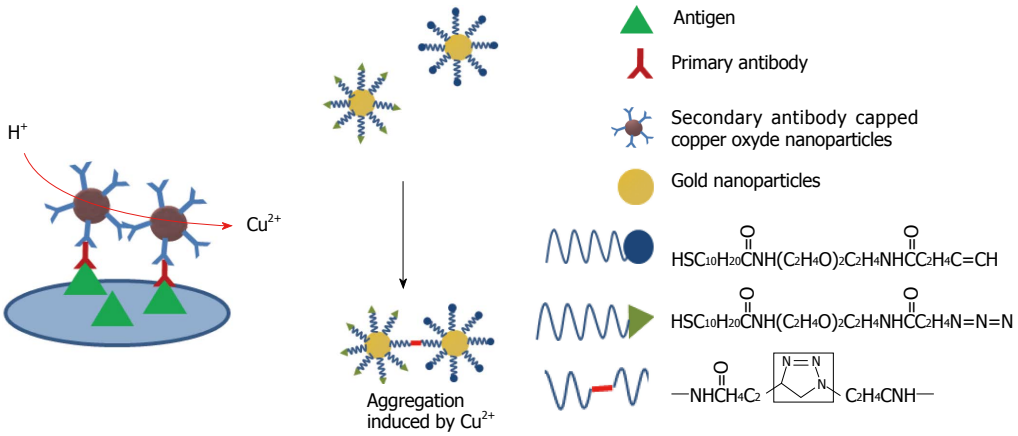


Figure 13 Schematic representation of immunoassay based on CuO-labeled antibody.

has received attention[102]. Silver nanoparticles are promising as the detection agent for electrochemical sensors. Porter highlighted the fact that although the number of assays reported with gold nanoparticles is much higher than those with silver, it is the silver nanoparticles that exhibit the better electrochemical properties[103]. Chen has developed the most sensitive electrochemical immunosensing method with a dynamic concentration range of 1-1000 ng/mL and a detection limit of 0.4 ng/mL[104].

The anti-microbial properties of silver are well documented[105], and the antimicrobial activity of silver nanoparticles dominates research in this area[106]. Recently, Singh reported the use of these hybrid compounds as anti-viral agents. The addition of silver nanoparticles to antibodies significantly increased the neutralizing potency in prevention of cell-associated human immunodeficiency virus (HIV)-1 transmission/infection (from 10% of inhibition for antibodies alone to 60%-71% for antibody capped silver nanoparticles)[107].

Copper: Since copper is easily oxidized, gold and silver nanostructures are more attractive for optical applications. In an effort to overcome this issue, research has been undertaken using different strategies. Zhang *et al*[108] proposed to cover the surface of copper nanoparticles with gold in order to combine the voltammetric activity of copper and the stability and biocompatibility of gold. The functionalization with antibody permits the detection of *E. coli* with a detection limit of 30 CFU/mL. More recently, Wang developed a method using antibody capped copper nanoparticles[109]. When Cu^{2+} is released into solution by HCl treatment, it can be assayed (Figure 13). The limit of detection for the GP41 glycoprotein of HIV was about 150 ng/mL.

Molecules from the family of supramolecular chemistry

Supramolecular chemistry concerns the domain of chemistry beyond that of the covalent bond and focuses on chemical systems made up of a discrete number of non-covalently assembled molecular subunits or components. The forces responsible for the spatial organization may vary from weak (intermolecular forces, van der Waals), through medium (aromatic-aromatic stacking or dipole-dipole) to strong (electrostatic, coordination bonds or hydrogen bonding)[110]. The assembly of the molecules will depend on molecular recognition events. Because of their recognition capability, molecules such cyclodextrins (CDs), calix[n]arenes, dendrimers, crown ethers or cucurbiturils have attracted interests as capping agents on metallic nanoparticles[111] (Figure 14).

Cyclodextrins: The CDs are a family of soluble molecules generally consisting of 6, 7 or 8 D-glucopyranosyl residues (denoted as α-CD, β-CD and γ-CD, respectively) linked in a cyclic structure by α-1,4 glycosidic bonds. They can form inclusion complexes incorporating various molecular guests within their hollow, truncated cone shaped cavity structures, enabling them to be used as drug carriers. The host-guest interactions involved have been attributed to a combination of weak interactions such as van der Waals forces, and hydrophobic interactions and stronger interactions including hydrogen bonds[112,113]. Their combination with metal nanoparticles was the first reported among the supramolecular family[114].

Gold: CD capped gold nanoparticles were initially investigated by Kaifer, in 1998, with the development of a new method based on the aqueous solubilization of aliphatic thiols by α-CD (Figure 15), which effectively binds to the aliphatic chains and carries the hydrophobic thiol molecules to the surface of the gold particles, where they undergo chemisorption[115]. This method can be used to prepare gold colloidal particles (diameter > 10 nm) modified with long chain alkanethiols. If the alkanethiol contains a bulky terminal group, such as ferrocene, the α-CD host is trapped after surface attachment, yielding CD-based rotaxanes supported on the gold nanoparticles. With the use of thiolated CDs, Liu *et al*[116] functionalized gold nanospheres (2-7 nanometers). The resulting monolayer-protected nanoparticles behave as multisite hosts in aqueous media, engaging in host-guest interactions with conventional guest molecules for CDs. Similarly, the group of Liu used the recognition ability of γ-CD for C60 fullerene, in order to create γ-CD

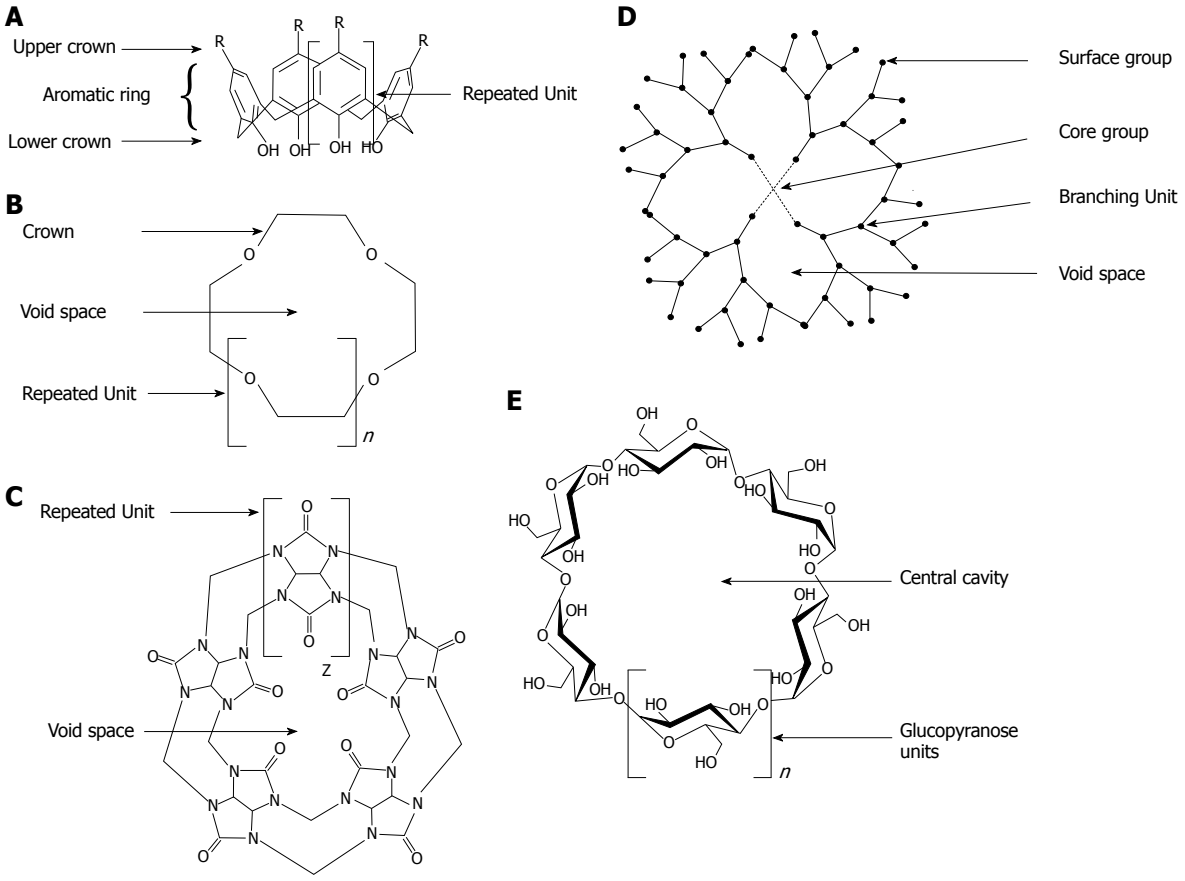


Figure 14 General structure of molecules from supramolecular family. A: Calixarene; B: Crown ether; C: Cucurbituril; D: Dendrimer; E: cyclodextrin.

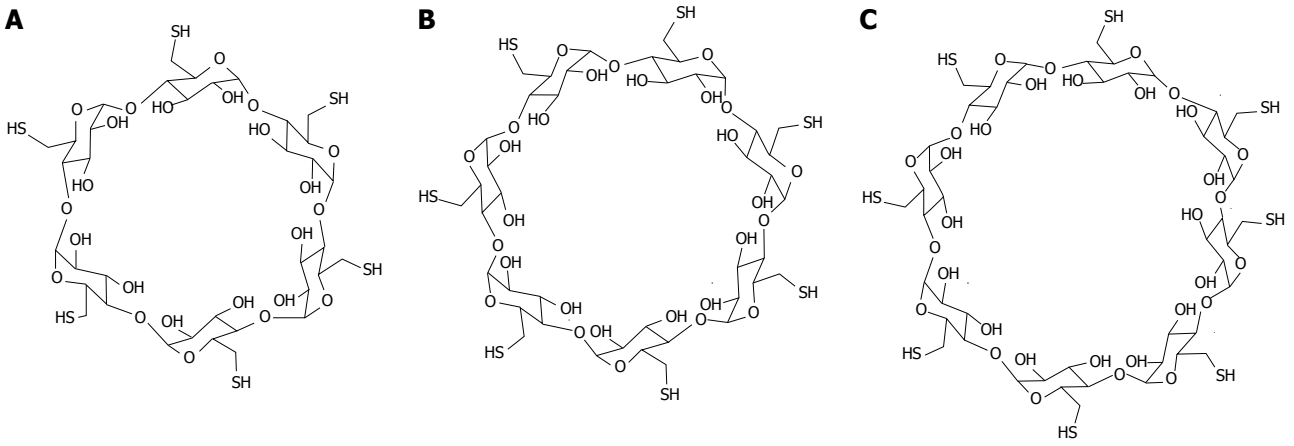


Figure 15 Structure of thiolated cyclodextrins. A: Per-6-thio-α-cyclodextrin; B: Per-6-thio-β-cyclodextrin; C: Per-6-thio-γ-cyclodextrin.

-capped gold nanoparticles as a C60 extracting agent. Even though C60 is extremely insoluble, an aqueous suspension containing γ-CD-capped gold nanoparticles (3.2 nm diameter) can partially solubilize C60[117]. This solubilization involves the formation of complexes between one molecule of C60 and two γ-CD hosts attached to different nanoparticles (Figure 16). Therefore, the fullerenes act as a sort of "molecular glue", leading to the formation of soluble nanoparticle aggregates with sizes around 290 nm.

A number of studies describe the use of CDs as capping agents for gold nanoparticles in order to construct nanoparticular superstructures in a reversible way[118,119]. In this area, Chen and Jiang have developed a reversible self-assembly of α-CD capped gold nanoparticles to vesicles, mediated by a guest (azobenzene) conjugated to the double hydrophilic block copolymers Poly-Isopropylacrylamide (PNIPAM) and poly dimethyl acrylamide[120]. This assembly mechanism occurs in pure water under the stimulus of temperature. A possible mechanism is *via* the thermal responsive coil-to-globule transition of the PNIPAM block (Figure 17).

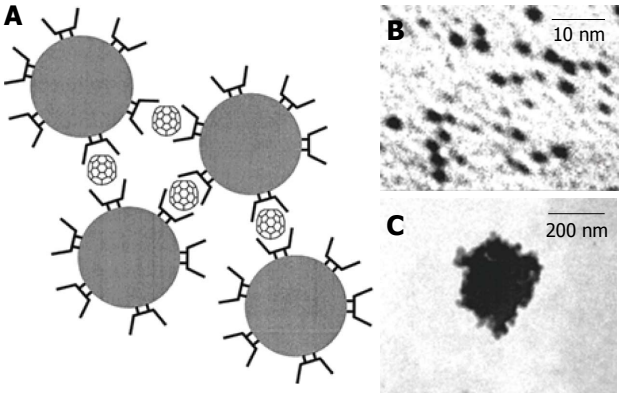


Figure 16 In A is represented a scheme of fullerene-induced network with cyclodextrin capped gold nanoparticles in aqueous solution, in B is shown Transmission Electronic Microscope image of dispersed cyclodextrin capped gold nanoparticles and in C is shown a Transmission Electronic Microscope image of fullerene-induced aggregate. From Liu *et al*[117], reproduced with permission from American Chemical Society Publications.

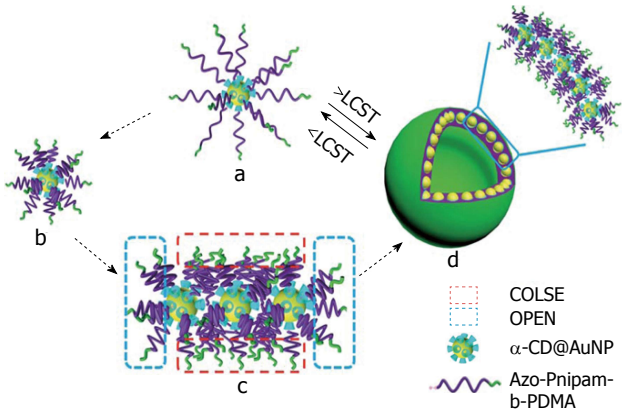


Figure 17 Schematic representation of a possible mechanism of Hybrid Inclusion Complex-vesicle formation. From Wei *et al*[120], reproduced with permission from Royal Society of Chemistry. CD: Cyclodextrin; PDMA: Poly dimethyl acrylamide; AuNP: Gold nanoparticle; LCST: Lower critical solution temperature.

Recently, CD-capped gold nanoparticles have been used as nanozymes[121]. They were utilized as a backbone to install metal catalytic centers by supramolecular assembly of the copper complex of triethylnetetramine-adamantane and 6-thio-β-CD 15.2 receptors immobilized on the gold surface by thiol groups. The catalytic behaviour of β-CD-15.2-modified gold nanoparticles with adjacent multi-metal catalytic centers were investigated as an esterase mimic. Strong hydrolase activity for cleavage of an active ester 4,4'-dinitrodiphenyl carbonate (DNDPC) was observed. The rate acceleration is approximately 2600-fold with CD-capped gold nanoparticles compared to the reaction rate for the non-catalyzed hydrolysis of DNDPC in the same buffer solution.

Silver: CD capped silver nanoparticles were developed more recently than CD capped gold nanoparticles, being first demonstrated by Fan[122]. The method is simple, addition of $NaBH_4$ to an aqueous solution of silver nitrate and α-CD. The authors explain the stabilization of the silver nanoparticles by hydrophobic interactions of α-CD primary faces. Then, hydrogen-bonding interactions between the exposed secondary -OH groups facilitates the threading of neighboring CDs, leading to the self-assembly of the silver nanoparticles into 1-D "pearl necklace" arrays.

Other studies have focused on the use of such hybrid systems to develop new antibacterial agents[123,124]. Wang developed silver nanoparticle-embedded one-dimensional β-CD-Poly-Vinyl-Pyrrolidone composite nanofibers using a one-step electrospinning technique. This composite exhibited good antibacterial properties against *E. coli* and *Staphylococcus aureus*[125].

CD capped silver nanoparticle have also been used as biosensors by using SERS[126] or visual inspection[127]. In the latter case, Chen described the development of a robust, colorimetric detection method, sensitive to different isomers of aromatic compounds. 6-thio-β-CD-15.2 was capped by chemisorbtion of the thiol function on 10 nm sized silver nanoparticles. The assay relies on the distance-dependent optical properties of silver nanoparticles and the different inclusion binding strength of the aromatic guests to CD. In the presence of different isomers of aromatic compounds, silver nanoparticles could be rapidly induced to aggregate, thereby resulting in an apricot to red colour change. With a spectrophotometer, this method is quantitative for monitoring the behaviour of the CD-modified silver nanoparticles as a function of the aromatic (Figure 18). The cited detection limit for different isomers of aromatic compounds is 5×10^{-5} mol/L.

Copper: CD based copper nanoparticles have been developed according to various methods. Zhu used 2-Hydroxypropyl-β-CD 4 (Figure 19) as a template to fabricate hollow spherical copper sulfide nanoparticle assemblies using sonication. The average size of the prepared copper sulfide nanoparticles was estimated to be 10 nm[128]. Geckeler reported the preparation of uniform copper oxide (tenorite) nanoparticles *via* a green pathway by thermal decomposition, using a novel supramolecular complex, in which β-CD is selected to encapsulate the precursor copper(Ⅱ) acetate[129].

Calix[n]arenes: The calix[n]arenes are one the most widely studied classes of organic supramolecular hosts[111]. This popularity arises from their ease of synthesis and the fact that they contain two very different chemistries, one at the phenolic functions and a second at the *para*-position on the aromatic ring. This is coupled with the possibility to region-selectively modify them with varying degrees of controlled substitution at either face (Figure 20). Finally, their molecular recognition abilities added to their lack of toxicities[130] have given calix[n]arenes many applications in biology from protein sensors, stabilizers, enzyme inhibitors to active pharmaceutical ingredient (API) solubilizers[131]. A secondary property that has proved highly advantageous is the propensity of the calix[n]arenes to crystallize, allowing solid-state studies of a wide range of their complexes with bio-active

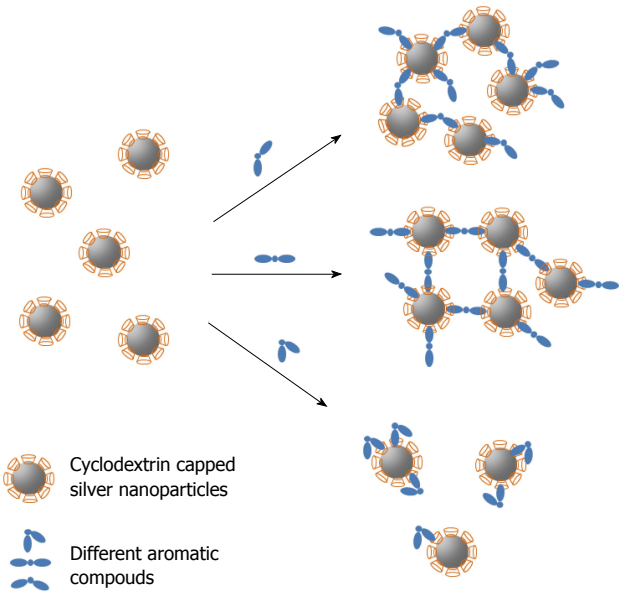


Figure 18 Schematic of host_guest recognition for Cyclodextrin capped silver nanoparticles with different aromatic compounds.

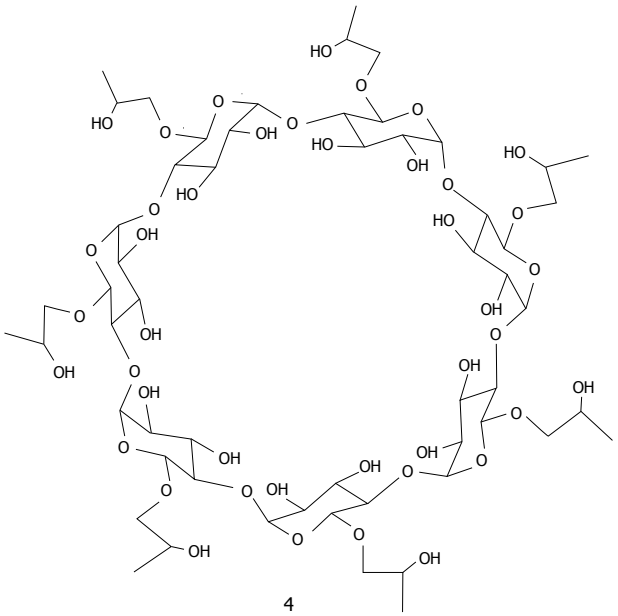


Figure 19 Structure of β-cyclodextrin derivative 4.

molecules[132]. Combined with the electronic and optical properties of metal nanoparticles, calixarene capped nanoparticles have already started to show new applications, as recently described[133,134].

Gold: So far, calixarene based gold nanoparticles have been mainly applied as colorimetric sensors. Pochini has carried out a large body of work in the use of thiolated derivatives of calix[4]arene for capping gold nanoparticles (Figure 21). The recognition of immobilized cationic pyridinium moieties with 5_GNP[135], or quaternary ammonium salts with 5_GNP or 6_GNP[136] by the unmodified upper rim of the calix[4]arene present on the gold nanoparticles was demonstrated.

Menon has introduced a simple route for the preparation of water soluble *para*-sulphonatocalix[4]arene thiol 7 capped gold nanoparticles[137] (Figure 22). 7 possesses an electron-rich cyclic cavity that can attract specifically cationic amino acids (lysine, arginine and histidine). These interactions between one amino acid molecule and two calix-modified gold nanoparticles tend to aggregate the assemblies more than the other amino acids (Figure 23). Similarly Han *et al*[138] used *para*-sulphonato-calix[6]arene 9, gold nanoparticles in order to detect pollutant aromatic amines isomers (Figure 24).

Other studies have focused on the recognition of cations. Yan *et al*[139] have developed 6 nm sized gold nanoparticles capped with methylthio-*para*-tert-butylcalixarene derivatives 11, 12 and 13 (Figure 25). They used the cationic recognition of structurally-tailored *para*-tert-butylcalixarenes to control access of the cationic guest to the cone cavity. This novel strategy has been shown to yield a specific red shift of the surface plasmon resonance band of gold nanoparticles, according to the cationic metal added. Solution of 0.4 μmol/L, calix GNP showed apparent rates of 1.4×10^{-2} s^{-1} for Cu^{2+} and 2.5 $\times 10^{-1}$ s^{-1} for Cs^{2+}.

Recently, Menon modified *para*-sulphonatocalix[4]arene with dithiocarbamate 14, for capping gold nanoparticles[140]. Sulfide ion recognition triggers particle aggregation through N-H-S hydrogen bonds and provides an easy way to measure color change (Figure 26). The lower detection limit was 10 nmol/L. This result validates the use of calix[n]arene capped gold nanoparticles in applications requiring high sensitivity and specificity.

Silver: Sanchez-Cortez used 25,27-diethyl-dithiocarbamic 26,28-dihydroxy *para*-tert-butylcalix[4]arene 15 in the functionalization of silver nanoparticles for pyrene detection by SERS[141]. SERS spectra provided information about the calix[4]arene orientation on the metal surface and the interaction mechanism (Figure 27).

Later Diao proposed a new method to change the surface properties of oleic acid stabilized silver nanoparticles and was successful in transferring silver nanoparticles from an organic phase into an aqueous phase[142]. By vigorous shaking of a biphasic mixture of the silver organosol protected with oleic acid and an aqueous solution of *para*-sulphonato-calix[4]arene 8, it is believed that an inclusion complex is formed between oleic acid molecules and 8, and the protective layer of the silver nanoparticles shifts from hydrophobic to hydrophilic in nature, which drives the transfer of silver nanoparticles from the organic phase into the aqueous phase. The 8-oleic acid inclusion complexation stabilized the nanoparticles for several weeks in the aqueous phase under ambient atmospheric conditions (Figure 28). Raston proposed new methods for environmentally friendly capping of silver nanoparticles with phosphonated derivatised calixarenes. *Para*-phosphonatedcalix[n]arenes 16, 17, 18 and 19 were used as stabilizers for evaluating the effect of hydrogen gas as an environmentally benign reductant of silver nanopar-

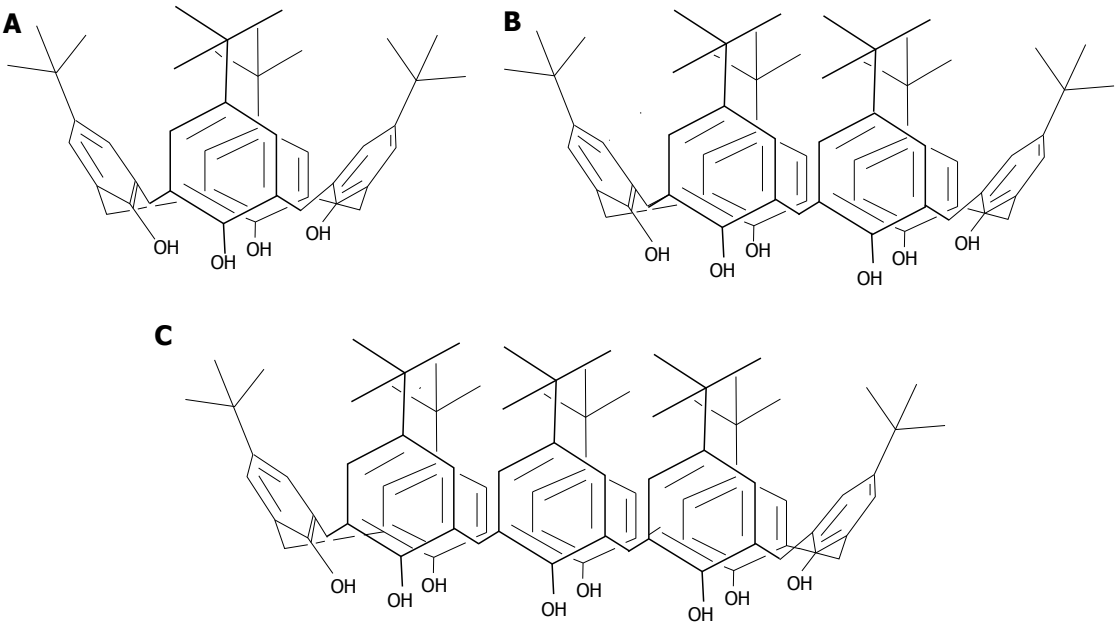


Figure 20 Structures of the t-Bu-calix[n]arenes, *n* = 4 (A), 6 (B) and 8 (C).

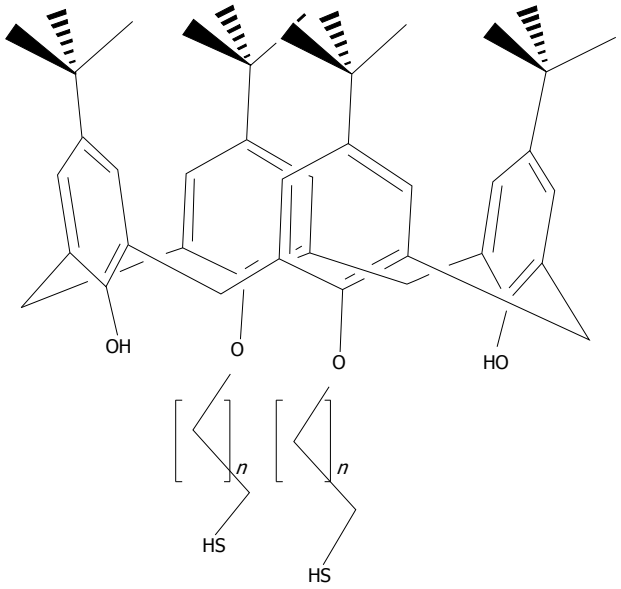


Figure 21 Structures of the thiolated-calix[n]arene derivatives. 5 corresponds to the present structure with *n* =10; 6 corresponds to the present structure with *n* = 5[136].

ticles[143] (Figure 29). Other phosphonated derivatized calixarenes 20 and 21 have been tested for possible their effect on the growth of silver nanoparticles by photochemical synthesis[144] (Figure 29).

It is the work of Li which has made popular calix[n]arene capped silver nanoparticles with his easy route for producing them[145]. The group demonstrated the application of 8 capped silver nanoparticles for the recognition of cationic amino acids (Histidine, Lysine, Arginine) and pesticides including methyl parathion[146].

Later, Coleman gave a more reasonable explanation for the structure of the assembly of 8 on the surface of the silver nanoparticles[147]. It lies in the formation of the classic bilayer solid-state structure, where alternate coordinated *para*-sulphonato-calix[4]arene molecules give available cavities at the surface of the nanoparticle (Figure 30). Such assembly allows differentiation between the interactions, nucleic acids and nucleotides with 8 capped silver nanoparticles. Subsequently, the group investigated the assembly of the calixarene with one nucleotide (cytosine) in different states: in solution, in the solid-state and on the surface of silver nanoparticle[148]. The assembly was quite different according to the states involved, and needed the use of multiple physical methods to probe the complex assembly process. Recently, the group of Coleman has shown that 8 capped silver nanoparticles could interact with active pharmaceutical ingredients[149]. More interesting was the use of calixarene capped silver nanoparticles for the determination of the Critical Micellar Concentration (CMC) of some cationic surfactants[150]. This method generates a new means of studying CMC in media containing proteins and in particular membrane proteins.

Copper: Interestingly, only one study from 2007 reports on the use of copper as material support for calix[n]arenes[151]. Uniform cuprous oxide nanospheres with diameter of 10 nm were prepared by the reduction of $CuSO_4$ in *para*-sulphonato-calix[8]arene 10 aqueous solution using hydrazine as a reducing agent. The host molecule, 10, was used as a bridge linker to make nanoparticles connect to each other and form large aggregations, which possess two types of properties: the photonic, catalytic and semiconductor properties of copper oxide and the supramolecular recognition function of 10-modified nanoparticles. As yet, there are no reports of any applications of this type of particle.

Dendrimers: Dendrimers are a class of hyper branched

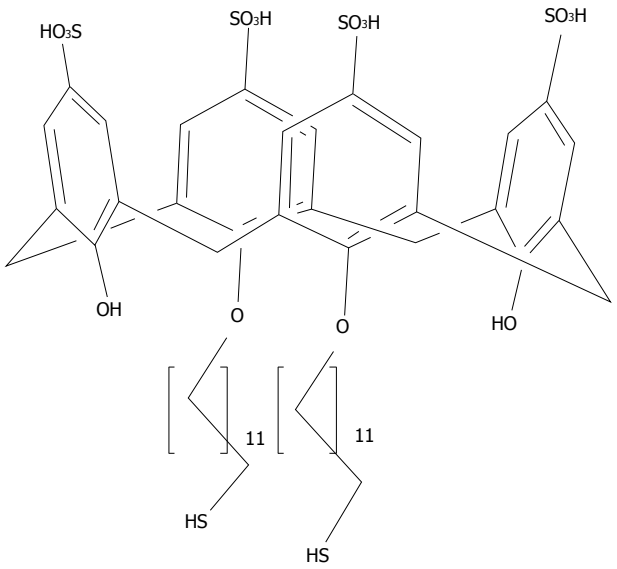


Figure 22 Structures of the *para*-sulphonato-calix[4]arene di-thiol 7.

oligomeric materials. Dendrimers are large and complex molecules having defined chemical structures. They possess three distinguishing architectural components, namely (a) an initiator core, (b) an interior layer (generations), composed of repeating units, radially attached to the initiator core and (c) exterior (terminal) functionality attached to the outermost interior generation (Figure 14). Dendrimers have been applied in biomedical applications, for drug-delivery systems and also for cancer therapy[152]. At this time, the major prospective applications of nanoparticle-dendrimer composites are in catalysis, biomedical research and electronic devices. The catalytic applications are mainly determined by properties of various mono- and bimetallic nanoparticles, while the dendrimer role is in templating or stabilization of nanoparticles, which, in turn, controls the nanoparticle size and morphology. There are a few examples where dendrimer generation (size) puts a limitation on the accessibility of the active centers for reacting molecules, due to a different size cavity in the dendrimer, thus creating size selectivity for a catalytic reaction. A similar effect was demonstrated for mesoporous catalysts with reactants of different sizes. The biomedical applications become possible due to the biocompatibility of many nanoparticle-dendrimer composites and their optical, magnetic and sensor properties[153].

In the last decade dendrimeric gold nanoparticle systems have been widely studied. Crooks was the first to first encapsulated small gold nanoparticles (1-2 nm of diameter) by using a thiolated fourth-generation poly (amido-amine) (PAMAM) dendrimer 22 mixed with tetrachloroauric acid and reduced with an excess of $NaBH_4$[154] (Figure 31). Crooks also used dendrimeric gold nanoparticles in catalysis including intradendrimer hydrogenation and carbon-carbon coupling reactions in water, organic solvents, biphasic fluorous/organic solvents and supercritical CO_2[155].

Lu *et al*[156] reported the use of RLS as a method for

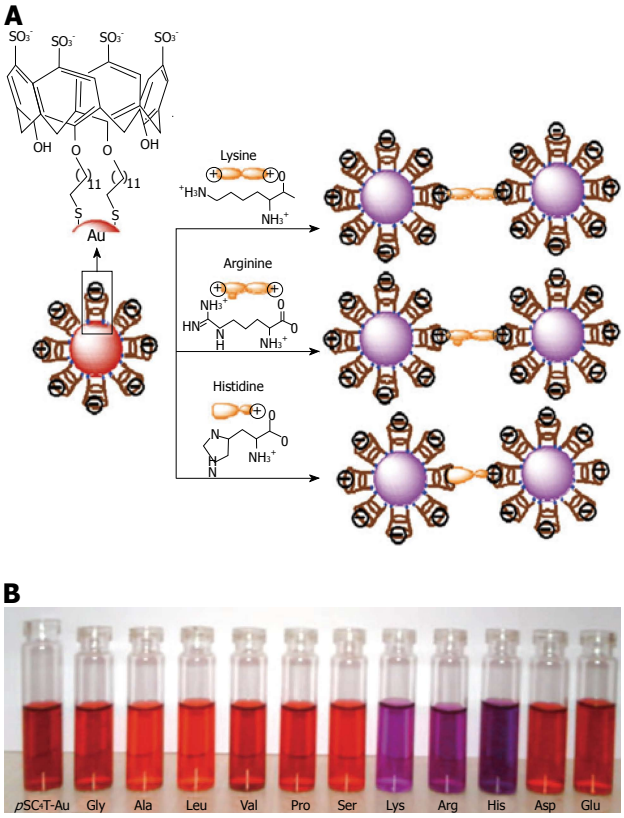


Figure 23 A is given a schematic representation of the amino acid induced aggregation of calix-capped gold nanoparticles; and in B are given the photographic images of calix-capped gold nanoparticles solutions containing different amino acids. From Patel *et al*[137], reproduced with permission from Royal Society of Chemistry.

detecting trace quantities of protein. Here, RLS measures the change of light intensity scattered from the gold nanoparticles. The signal is known to be amplified upon aggregation. Generation of polypropylene imine hexadecane amine dendrimers 23 (PPIHA) was employed to synthesize uniform gold nanoparticles modified with amine groups on their surface (Figure 32). The amine groups strengthen the covalent coupling between gold nanoparticles and bovine serum albumin (BSA). As illustrated in Figure 33, the size of the gold nanoparticle-BSA conjugates was increased in the $HAuCl_4$-NH_4OH_HCl reaction system, enhancing the RLS intensity of the bioconjugates. The RLS intensity is related to the concentration of gold nanoparticle-BSA and has a lower detection limit of 0.090 μg/mL. By employing BSA as a model protein, this work introduced a novel method for the quantitative detection of trace proteins[157]. Recently, Baker has developed a simple approach to fabricating multifunctional dendrimer-stabilized gold nanoparticles for cancer cell targeting and imaging[158]. In this work, amine-terminated generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers pre-functionalized with folic acid and fluorescein isothiocyanate are complexed with Au (Ⅲ) ions, followed by acetylation of the amine groups on the dendrimer surfaces. This one-step process leads to the spontaneous formation of 6 nm Au nanoparticles stabilized by

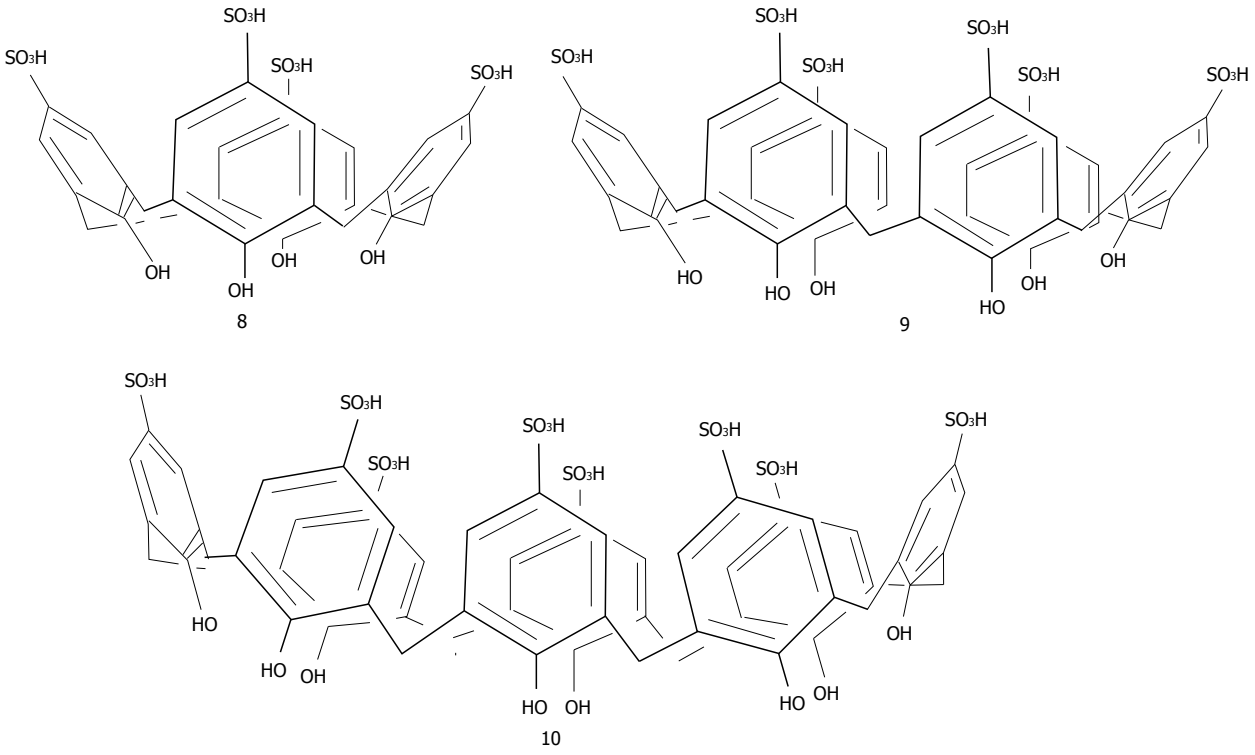


Figure 24 Structures of the para-suphonato-calix[4, 6 and 8]arenes.

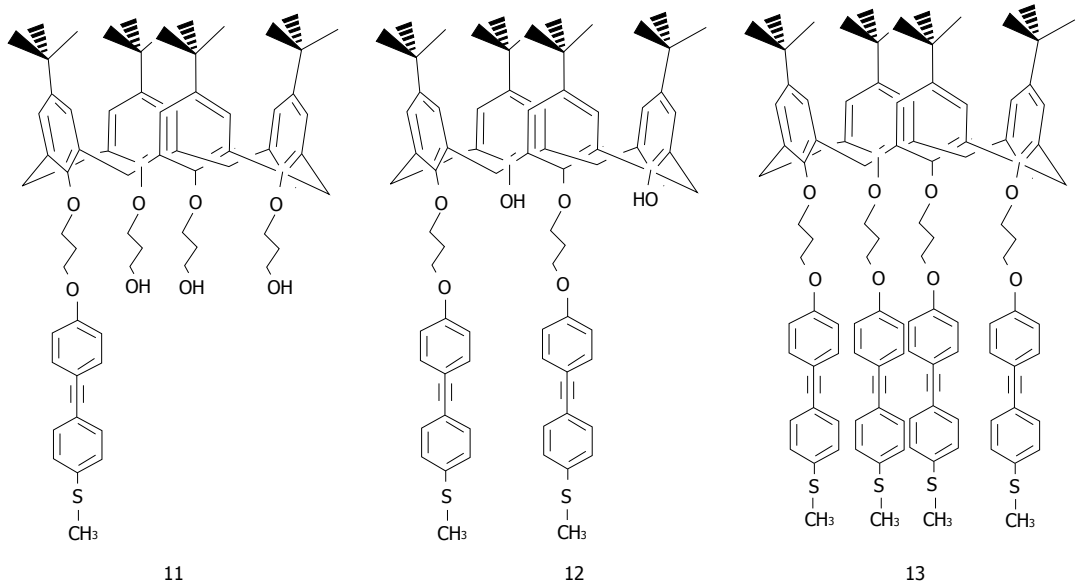


Figure 25 Structures of the methylthio-para-tert-butyl-calixarene derivatives.

multifunctional dendrimers bearing both targeting and imaging functionalities.

Wang used dendrons, segments of dendrimers that possesses a focal point onto which the branching units of a dendritic architecture are attached to associated gold nanoparticles[159] (Figure 34). It was demonstrated that dendrons modified with a metal-coordinating functionality can be utilized as stabilizing media for the controlled growth of nanocrystals. The average size of the resulting nanoparticles is a direct function of the generation number of the capping dendron, with higher generation dendrons producing larger particles. Astruc has made a large contribution in the research and development of dendronized gold nanoparticles[160]. Dendrons have been synthesized and used to assemble dendronized gold nanoparticles either by the ligand-substitution method from dodecanethiolate-gold nanoparticles (AB3 units) or Brust-type direct synthesis from a 1:1 mixture of dodecanethiol and dendronized thiol (AB9 units). Two nanoparticles have been made containing a nonasilylferrocenyl dendron 24 and 25 (Figure 35)[161], bear ing respectively 180 and 360 ferrocenyl units at the periphery.

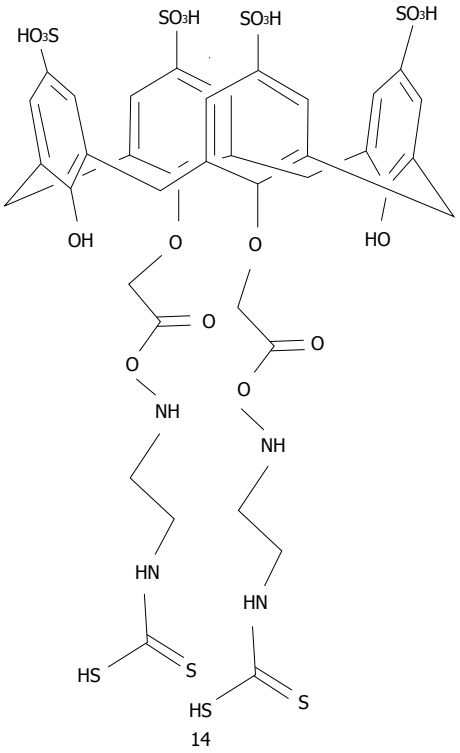


Figure 26 Structure of 25,27-bis(ethyleneaminecarbonylmethoxy)-26,28-dihydroxy-*para*-sulphonatocalix[4]arene modified dithiocarbamate.

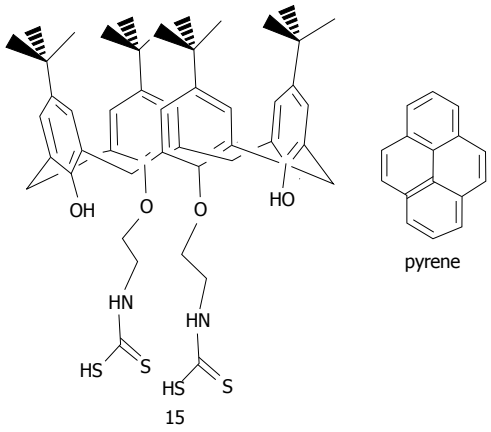


Figure 27 Structures of 25,27-diethyl-dithiocarbamic acid 26,28-dihydroxy *para*-tert-butylcalix[4]arene 15 and pyrene[141].

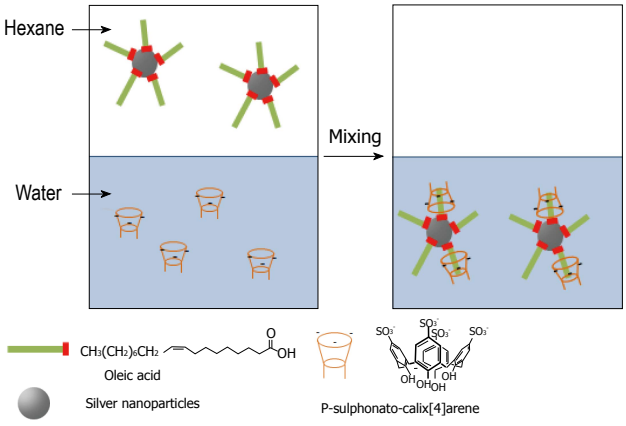


Figure 28 Phase transfer of Oleic acid stabilized silver nanoparticles from hexane to para-sulphonato-calix[4]arene aqueous solution.

These colloids selectively recognize the anions $H_2PO_4^-$ and adenosine-5'-triphosphate (ATP^{2-}). Recognition has been monitored by cyclic voltammetry.

Silver: Recently Kakar reviewed dendrimer templated construction of silver nanoparticles. Up to now, synthesis assisted by dendrimers has led almost exclusively to formation of spherical-shaped silver nanoparticles. It is believed that dendrimers are likely to be more valuable for modulating the size of silver nanoparticles than their shape[162].

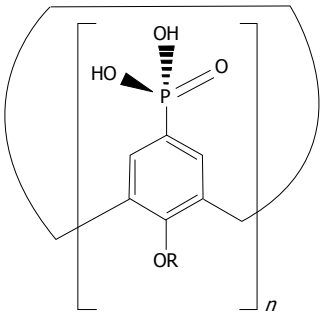
Balogh *et al*[163] reported that PAMAM Dendrimer 23 attached silver nanoparticles display considerable activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli* bacteria without the loss of solubility and activity in the presence of sulfate or chloride ions. Balogh has shown that dendrimer silver nanoparticles may find potential application as cell biomarkers[164]. They have synthesized hydroxyl-, and carboxyl-terminated ethylenediamine core generation 5 poly(amidoamine) dendrimers which were utilized to prepare aqueous silver-dendrimer nanoparticles. The hybrid particles are water-soluble, biocompatible, fluorescent, and stable below pH 7.5 The cellular uptake of nanoparticles was examined by transmission electron microscopy and confocal microscopy. Overall, cytotoxicity analysis indicates that the uptake of the hybrid particles is correlated with the surface charge of dendrimer and that the silver has no effect.

Mali reported the synthesis and characterization of a novel electrochemical label for sensitive electrochemical stripping metallo-immunoassays based on silver dendrimer-encapsulated nanoparticles[165]. Several fixed ratios of Ag^+/dendrimer were prepared with the aim of obtaining stable nanocomposites with maximal silver loading in the interior of a polymeric shell. By combination of differential pulse voltammetry and anodic stripping analysis on a carbon electrode, individual silver dendrimer-encapsulated nanoparticles (limit of detection is 0.9 pMol) were detected down to 1.35×10^{10} after the dissolution of silver nanoparticles in dilute nitric acid.

Dendrimer silver nanoparticles have been shown to be good catalysts for reactions, such as the reduction of nitrophenol[166], chloronitrobenzene[167] or the 2,7-dicholoroflurescein dye[168].

Copper: Various methods can be found in the literature to produce dendrimer copper nanoparticles, ranging from classical metal reduction using dendrimer as stabilizer[169] to more original electrochemical preparation[170].

Using molecular assembly properties, Moore showed that dendrimer associated copper nanoparticles could be used as a catalyst of Cu^+-catalyzed azide-alkyne cycloaddition[171]. Reactivity was tested on a model reaction between azido propanol and propargyl alcohol in aqueous solution. The authors observed up to 120 fold faster con-



16 R = H, *n* = 4

17 R = H, *n* = 5

18 R = H, *n* = 6

19 R = H, *n* = 8

20 R = C_8H_{17}, *n* = 4

21 R = $C_{10}H_{21}$, *n* = 4

Figure 29 General structures of para-phosphonato-calix[n]arene derivatives.

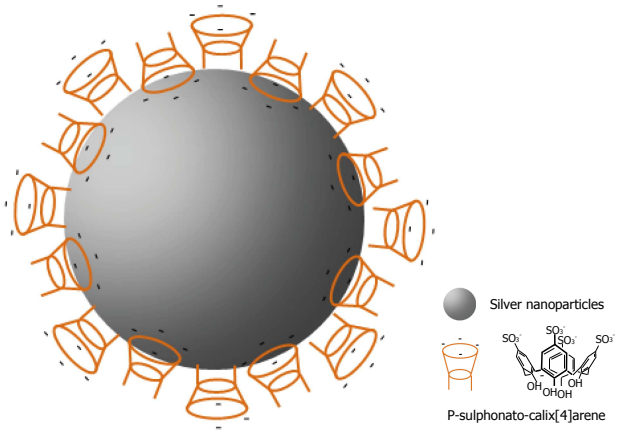
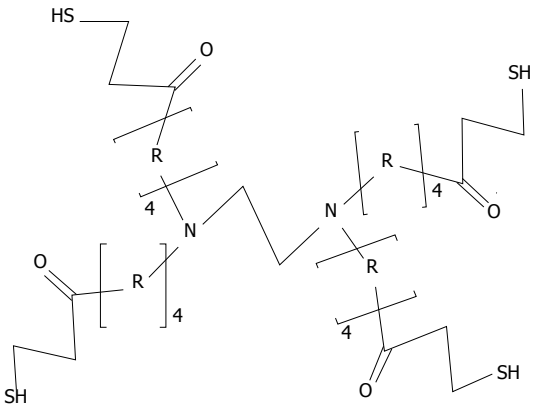


Figure 30 Schematic representation of the organization of *para*-sulphonato-calix[4]arene, 8 on silver nanoparticles.

version using PAMAM dendrimers as macromolecular Cu^+ ligands compared to traditional small molecular ligand systems, and demonstrated that the macromolecular catalyst can be removed by ultrafiltration.

Huang has successfully synthesized mono-, di-, and tri-functionalized G5 PAMAM dendrimer conjugates with a copper-free click conjugation method[172]. An azido modified targeting moiety, a therapeutic drug and an imaging reagent were mixed with a G5 PAMAM dendrimer nanoplatform, simultaneously or sequentially, to give mono-, di- and tri-functional conjugates.

Crown ethers: Since Pedersen first reported the synthesis and cation complexation properties the crown ethers in 1967, these neutral synthetic heterocyclic compounds have attracted extensive and continuous attention through their unusual and powerful non-covalent cation binding properties. Classical crown ethers are macrocyclic polyethers that contain 3-20 oxygen atoms, each separated from the next by two or more carbon atoms (Figure 14)[173]. The most effective complexation agents, however, are macrocyclic oligomers of ethyleneoxy units, either substituted or unsubstituted, that contain 5-10 oxygen atoms. They are exceptionally versatile in selectively, binding a range of metal ions and a variety of organic neutral and ionic species. Crown ethers are currently being studied and used in a variety of applications beyond



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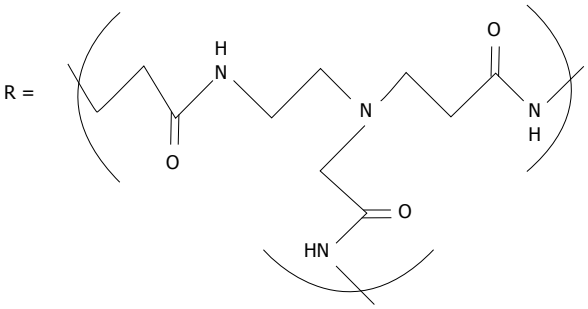

Figure 31 Structure of generation 4 thiolated dendrimer studied by Crooks[155]. The repeating unit R corresponds to poly(amido-amine).

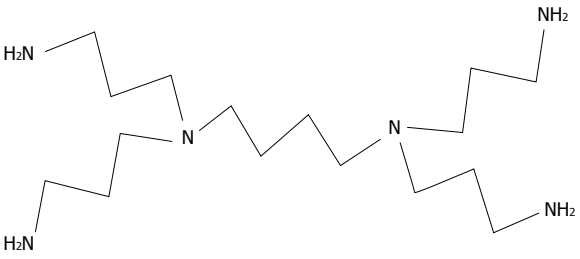

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Figure 32 Structure of polpypropylneiminehexadecaneamine (sic) dendrimer (PPHA generation 1st).

their traditional place in chemistry (used in the laboratory as phase transfer catalysts). In the biological context, they are being investigated as a promising anti-cancer compound[174].

The combination of crown ethers with metallic nanoparticles has been mainly employed as a cation sensor. So far, crown ether capped gold nanoparticles have been used for cation detection using dithiocarbamate modified *N*-benzyl-4-aminobenzo-15-crown-5-ether 26 for K^+[175,176] or aza-15-crown-5-ether acridinedione 27 for Ca^{2+} and Mg^{2+}[176] (Figure 36). Li *et al*[177] used silver because of its higher optical extinction ratio (stronger than gold). He capped dithiocarbamate modified aza-15-crown-5-ether 28 on silver nanoparticles with an average size of 8 nm of diameter (Figure 37). Li was able to detect Ba^{2+} with a detection limit of 1×10^{-8} mol/L. A possible explanation of the aggregation induced with Ba^{2+} is given by the formation of the sandwich structure with crown

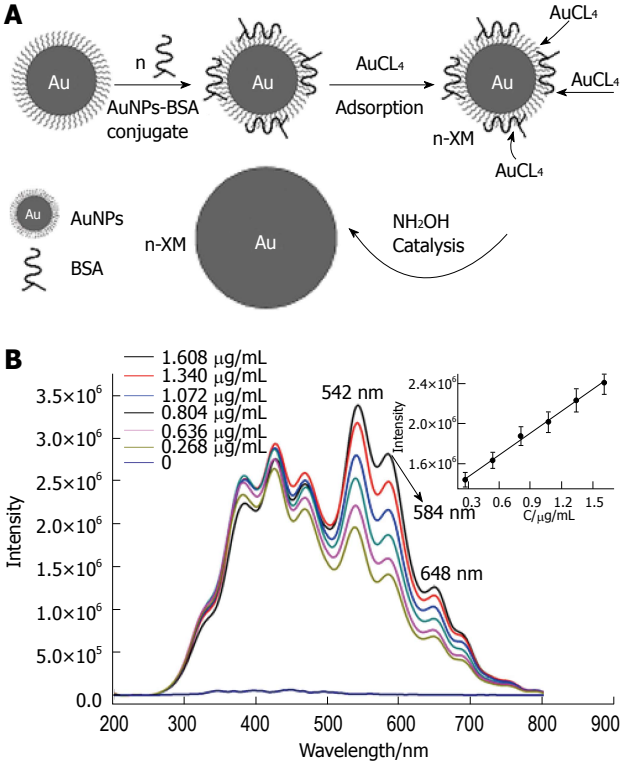

Figure 33 In A is given a schematic representation of the resonance light scattering amplification assay of biomolecules based on the biomineralization of gold nanoparticles bioconjugates; in B is given the resonance light scattering spectrum of the biomineralization product of concentration gradient of gold nanoparticles bioconjugates. From Liu *et al*[157], reproduced with permission from Elsevier. BSA: Bovine serum albumin; AuNP: gold nanoparticle.

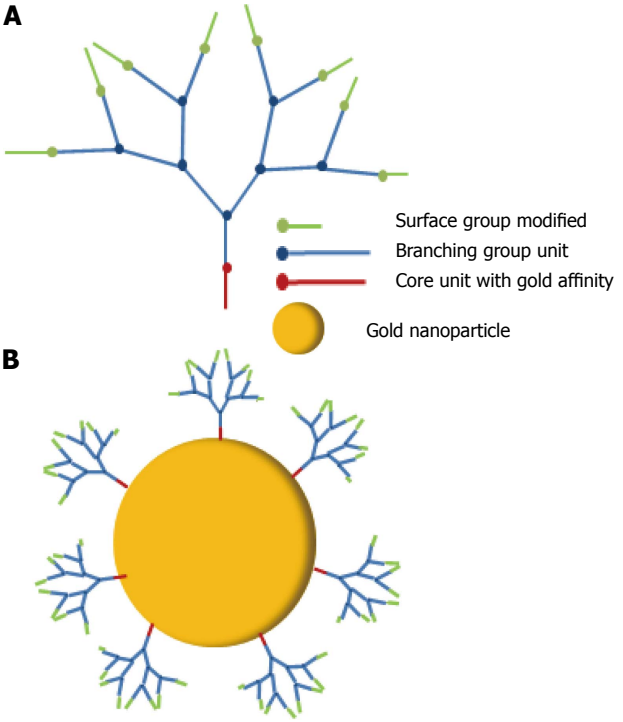

Figure 34 Schematic representation of dendron generation 3 (A) and a dendron generation 3 capped gold nanoparticle (B).

ether (Figure 38).

Kwang has developed a colorimetric method of melamine detection, based on 18-crown-6 ether 29 (thiol derivatized) functionalized gold nanoparticles with an average diameter of 20 nm[178] (Figure 39). Melamine is a plant metabolite of cyromazine pesticides and a common chemical. It is also a highly toxic agent used fraudulently in the food industry. Crown ether capped gold nanoparticles enables the detection of melamine in milk after a pre-treatment consisting of centrifugation and purification. The crown ether GNP aggregation induced by the melanine is then monitored by UV-visible spectra with a LOD as low as 6 ppb, a wide linear range from 10 to 500 ppb, and acceptable reproducibility and specificity. This method could be extended to other toxins which show sufficient specificity in relation to the crown ethers and assembly ability (creating bridge between nanoparticles for aggregation).

Cucurbiturils: Cucurbiturils (CBs), are macrocycles derived from glycoluril units, which form stable host-guest complexes with various guest cationic molecules (Figure 14). Cucurbiturils have gained attention due to their unique structure and multiple recognition properties, as well as their potential applications for constructing sensors, drug delivery and biomimetic systems. Geckeler and collaborators initially reported a simple, green, one-pot synthesis of well-dispersed CB capped silver nanoparticles by the reaction of an aqueous silver nitrate solution with CB[7] in the presence of NaOH at room temperature[179]. Furthermore, they have investigated the *in vitro* cytotoxic properties of the prepared silver nanoparticles against two different human cancer cell types, namely human breast adenocarcinoma (MCF-7) and human lung bronchoalveolar (NCIH358) cells. It was demonstrated that the prepared CB[7]-protected silver nanoparticles, with an average size of about 5 nm, could be suitable candidates for cancer therapy applications. Mason prepared a series of CB capped silver nanoparticles and aggregates by reduction of silver nitrate with sodium borohydride in the presence of different CB[180]. They addressed the impact of CB[n] macrocycles (n = 5-8) on the formation and stabilization of aqueous silver nanoparticles and Ag nanoaggregates obtained from silver nitrate and sodium borohydride, in the absence and presence of a set of positively charged guests shielding one or both portals of the cavitand. While CB[5] and CB[6] caused rapid aggregation and precipitation of Ag aggregates (diameters >13 nm), CB[7] and CB[8] allowed the formation and stabilization of monocrystalline, narrowly dispersed silver nanoparticles (diameters 5.3 and 3.7 nm, respectively). This could be explained by the rigidity of CB[5] and CB[6], and their possible lack of suitable arrangement at the silver surface, giving a poor stabilization of these silver assemblies, while the more flexible CB[7] and CB[8] may undergo some minor distortions and better adapt to the requirements of the metallic surface.

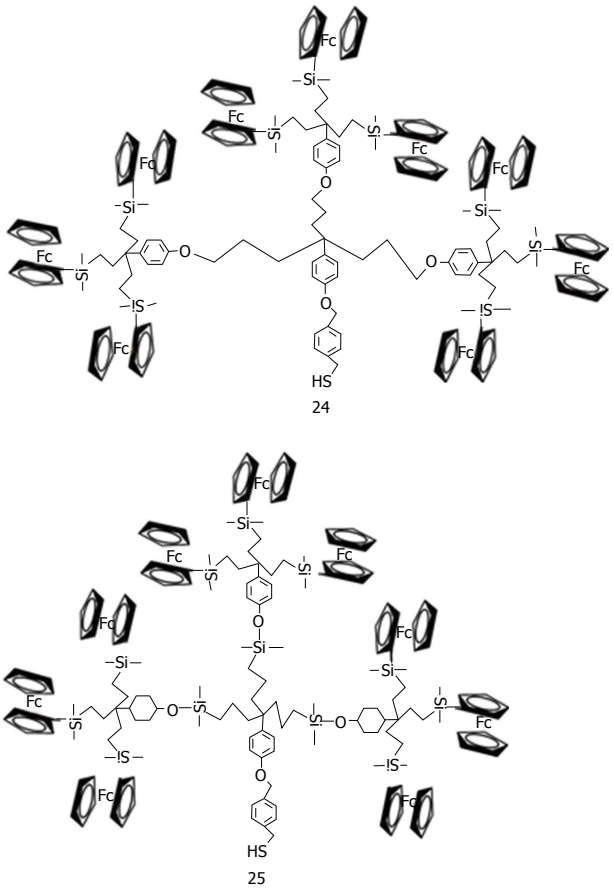

Figure 35 Structures of nonasilylferrocenyl dendron deriratives studied by Astruc *et al*.

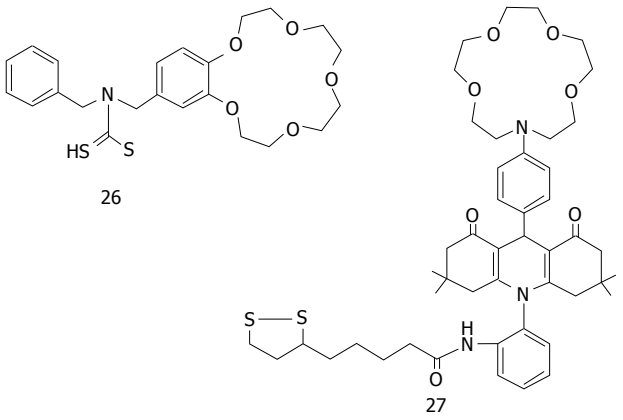

Figure 36 Structures of N-benzyl-4-aminobenzo-15-crown-5ether 26 for K^+ and aza-15-crown-5-ether acridinedione 27.

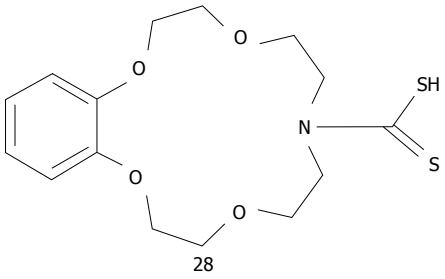

Figure 37 Structures dithiocarbamate modified aza-15-crown-5-ether 28 studied by Li *et al*.

TOXICITY CONCERNS

Since hybrid nanoparticles have been investigated as therapeutic agents, a number of studies have investigated their toxicity[181]. More importantly, because of their numerous applications (described above) already high level of production of hybrid nanoparticles is growing and this inevitably leads to their appearance in air, water, soil and organisms[182]. As a consequence the risks to the environment and to human health are significant and should be treated as a concern.

Ray reviewed the different effects on health of different metal nanoparticle preparations[183]. Nanotoxicity studies revealed that the physicochemical characteristics of engineered nanomaterials play an important role in their interactions with living cells[184]. Physicochemical properties that affect the biological activity of hybrid nanoparticles include particle size, shape, surface chemistry, surface area, surface charge and their metallic composition[185].

Gold

While bulk gold is considered as "safe", nanoscale particles of gold need to be examined for biocompatibility and environmental impact if they are to be manufactured on a large scale for *in vivo* usage[186]. Several researchers have reviewed the toxicity of gold nanoparticles in cells (cytoxicity) and *in vivo*[187,188]. Murphy reviewed these studies and highlighted some key parameters in the toxicity process[189]. First, the cell type is of critical importance. Patra studied the cell selective response to gold nanoparticles[190] and found that nanoparticles induced death in the A549 human carcinoma lung cell line while two other cell lines tested, BHK21 (baby hamster kidney) and HepG2 (human hepatocellular liver carcinoma), remained unaffected. The second key parameter is the surface charge. Cationic nanoparticles are much more cytotoxic than anionic particles. This may be related to their electrostatic interaction with the negatively charged cell membrane[191]. Size and shape are further critical parameters regarding potential toxicity. Chithrani *et al*[192] observed that the cellular uptake of gold nanoparticles was greatly size dependent. Spheres of 50 nm were taken up more quickly by cells than either smaller or larger spheres in the 10-100 nm range and spheres were taken up more efficiently than nanorods that had dimensions in the 10-100 nm range[193]. Dikman has reviewed recently *in vivo* studies concerning gold nanoparticles and came to three conclusions[193]. Firstly, the dose and possible inflammatory processes are of paramount importance for the clearance (process avoiding accumulation in the organs of the reticuloendothelial system, such as spleen or liver) of 10-100 nm gold nanoparticles; Secondly, the effect of nanoparticle penetration *via* the hematoencephalic barrier depends critically on their size; 5-20 nm being the upper limit; Thirdly, gold nanoparticles of 1-2 nm in diameter

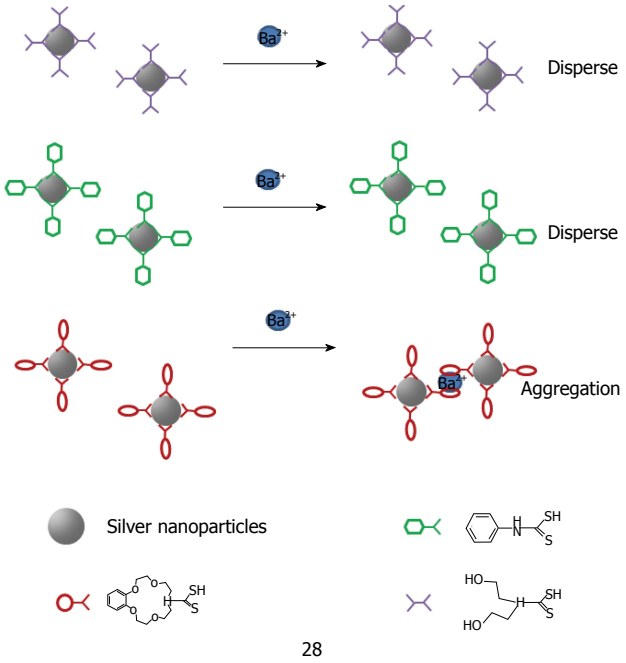

Figure 38 Schematic illustration of the aggregation of crown ether capped silver nanoparticles in the presence of metal ions Ba^{2+}.

could be more toxic due to the possibility of irreversible binding to biopolymers in cells. Also, numerous experiments on cell cultures have revealed no observable toxicity in colloidal particles with a size of 3-100 nm.

Silver

Silver was originally used as an effective antimicrobial agent and as a disinfectant, as it was relatively free of adverse effects[194]. However even if silver is believed to be relatively nontoxic to mammalian cells, many *in vitro* studies have been performed to determine if this is still the case for silver nanoparticles. Several reviews summarize their effect on cells[195-197] *in vitro* studies have shown that silver nanoparticles have potential to induce toxicity in cells derived from a variety of organs. Chueh proposed a mechanism of cytotoxicity for fibroblast cells[198]. Silver nanoparticles induce cellular death (called apoptosis) by the generation of reactive oxygen species (ROS) and the activation of a specific biochemical pathway, JNK, *via* the mitochondria.

Despite the fact that silver nanoparticles have been increasingly applied in the biomedical and pharmacological fields, relatively little research has been done into their possible side-effects in clinical medicine[197]. Silver ingestion and topical application can induce the benign condition known as "argyria", a grey-blue discoloration of the skin and liver caused by deposition of silver particles in the basal laminae of such tissues. Although argyria is not a life-threatening condition it is, however, cosmetically undesirable[199]. Wong *et al*[200] reviewed and themselves investigated the effect on health of silver nanoparticles. They injected silver nanoparticles intravenously into experimental mice and did not observe any overt systemic effects, despite the silver nanoparticle solution used being at the relatively high concentration of 100 mmol/L. With to this administration route and dose application, silver nanoparticles can be potentially toxic. Intravenous administration may have more implications for adverse effects than dermal application[195].

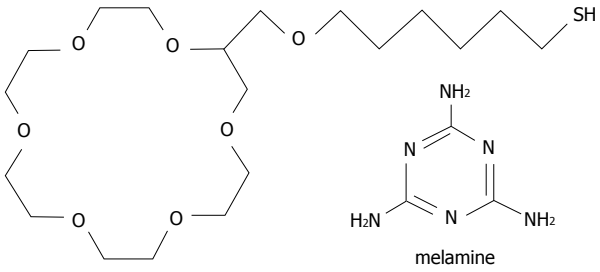

Figure 39 Structures of thiolated crown ether 29 and melamine studied by Kuang *et al*.

Copper

Compared to gold and silver, the cytotoxicity of copper has been less studied[83]. However, Kim has recently investigated the toxicity of different metal nanoparticles[201]. They used the laser ablation method to generate Ag, Au, Co and Cu NPs in biocompatible aqueous solution. This method consists of intense laser irradiation of a material in solution and leads to an ejection of its constituents and the formation of nanoparticles[202]. This method allows generation of uncapped nanoparticles, allowing investigation of the toxicity of the metal itself. The cytotoxicity assay results demonstrate that nanoparticles possess moderate cytotoxicity to human cells in a cell-dependent manner. The half maximal inhibitory concentration (IC_{50}) has been respectively determined for Hela and PC3 cell lines: 78.9 and 88.6 μg/mL of silver NP; 66.4 and 82.9 μg/mL of gold NP and 85.5 and 91.7 μg/mL of copper NP. Interestingly, the copper nanoparticles showed the lowest cytotoxicity. Valodkar produced 10 nm water soluble copper nanoparticles using starch as stabilizing agent and ascorbic acid as reducing agent[203]. These nanoparticles showed relatively low cytotoxicity (half maximal lethal dose of 100 μg/mL) and a strong bactericidal effect. The Minimum Bactericidal Concentration, the lowest concentration of antibiotic required to kill a particular bacterium, was determined as 3.2 μg/mL for *S. Aureus* and 1.6 μg/mL for *E. coli*. Because of their bactericidal effect at non-cytotoxic dose, these copper nanoparticles seem promising as possible therapeutic agents. However, clinical studies on silver and gold nanoparticles are required before considering copper nanoparticles as safe.

CONCLUSION

In this review we have presented the preparation and physical properties of Noble metal capped nanoparticles. All aspects of molecular recognition of the various types of capping agent derived nanoparticles have been discussed in depth. Finally notice has been taken of the

concerns of many people with respect to the possible toxic effects of capped Noble metal nanoparticles.

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P- Reviewer Tanaka T **S- Editor** Wen LL **L- Editor** A **E- Editor** Yan JL

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
wjbc@wjgnet.com
doi:10.4331/wjbc.v4.i3.64

World J Biol Chem 2013 August 26; 4(3): 64-70
ISSN 1949-8454 (online)

MINIREVIEWS

Autophagy and cancer

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Received: June 29, 2013 Revised: July 13, 2013
Accepted: July 17, 2013
Published online: August 26, 2013

Abstract

Autophagy is a homeostatic and evolutionarily conserved mechanism of self-digestion by which the cells degrade and recycle long-lived proteins and excess or damaged organelles. Autophagy is activated in response to both physiological and pathological stimuli including growth factor depletion, energy deficiency or the upregulation of Bcl-2 protein expression. A novel role of autophagy in various cancers has been proposed. Interestingly, evidence that supports both a positive and negative role of autophagy in the pathogenesis of cancer has been reported. As a tumor suppression mechanism, autophagy maintains genome stability, induces senescence and possibly autophagic cell death. On the other hand, autophagy participates in tumor growth and maintenance by supplying metabolic substrate, limiting oxidative stress, and maintaining cancer stem cell population. It has been proposed that the differential roles of autophagy in cancer are disease type and stage specific. In addition, substrate selectivity might be involved in carrying out the specific effect of autophagy in cancer, and represents one of the potential directions for future studies.

Key words: Autophagy; Cancer; Cancer stem cells; Therapy resistance; Mitophagy; BNIP3

Core tip: The differential expression of selective autophagic receptors in cancers of different origin and stage might induce the selective removal or preservation of certain cellular components and contribute to either tumor suppression or cancer cell survival.

Lu SZ, Harrison-Findik DD. Autophagy and cancer. *World J Biol Chem* 2013; 4(3): 64-70 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i3/64.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i3.64

INTRODUCTION

Autophagy is an evolutionarily conserved catabolic pathway which delivers long-lived proteins and excess or damaged organelles into the lysosome for degradation and recycling[1,2]. Three mechanistically distinguished subtypes including macroautophagy, microautophagy and chaperon-mediated autophagy exist, of which, macroautophagy (hereafter referred to as autophagy) is most studied. Traditionally known as a mechanism to maintain homeostasis and degrade cellular components in response to starvation, further functions have been identified as our understanding of autophagy has progressed. A novel role of autophagy in cancer has also been proposed in recent years. In the current review, we attempt to provide a brief evaluation of the current literature and discuss the potential mechanisms of how autophagy is involved in the pathogenesis of cancer.

AUTOPHAGY MACHINERY AND REGULATION

The basic machinery and regulation of autophagy has

been described in numerous excellent reviews[1,3-6] and will not be discussed in detail here. We will briefly introduce the autophagy process and key players to facilitate our further discussion. Autophagy process is divided into four stages: nucleation, elongation, autophagosome formation and fusion. The nucleation is initiated by the dephosphorylation (*i.e.*, activation) of the unc-51-like kinase (ULK) complex. ULK complex is otherwise kept inactive by the mammalian target of rapamycin (mTOR), a highly conserved serine/threonine protein kinase. mTOR integrates the signal of growth factor and nutrition availability and serves as the pivotal inhibitory regulator of autophagy. In other words, limited growth factor and nutrient inactivates mTOR and release ULK complex from its inhibition. Upon activation, ULK complex induces the re-localization of a phosphatidylinositol-3-kinase-class Ⅲ (PtDIns3K) complex, which is composed of vacuolar protein sorting 34 (Vps34), p150, mAtg14 and Beclin1, to the nucleation site. Beclin1 mediates the cross-talk between autophagy and apoptosis in that it is a binding partner of anti-apoptotic Bcl-2 family proteins (*e.g.*, Bcl-2, Bcl-xl and Mcl-1). Beclin1 can be sequestered by these Bcl-2 proteins, which will prevent the formation of PtDIns3K complex and thereby block the nucleation process. The pro-apoptotic BH-3 only Bcl-2 proteins (*e.g.*, Bnip-3, Bad and Puma) compete with Beclin1 for the binding to anti-apoptotic Bcl-2 proteins and hence promote autophagy. Once formed, PtdIns3K complex catalyzes the production of phosphatidylinositol (3)-phosphate [PtdIns(3)P], which further recruits autophagy related (Atg) proteins. The two interrelated ubiquitin-like conjugation systems, Atg12-Atg5-Atg16 and microtubule-associated protein light chain 3 (LC3)-phosphatidylethanolamine (PE) play a major role in the elongation of the phagophore. The subsequent step, autophagosome formation, is accomplished by the invagination of phagophore membrane and the sequestration of cytosolic contents. In order for its contents to be degraded, the autophagosomes will form autolysosomes by fusing with lysosomes or late inner body.

SUBSTRATE SPECIFICITY OF AUTOPHAGY

In recent years, the concept of substrate selectivity in autophagy has gained further recognition. This is quite different from the initial understanding of autophagy, which was regarded as a non-specific self-eating process. However, recent studies have indicated that a specificity for substrate in autophagy is conveyed through different receptor proteins. More importantly, a correlation between the targeted removal of cellular components by autophagy and human diseases has been established[7]. The autophagy receptors, which play a key role in the substrate selectivity[8], tether the substrate of interest to the autophagic machinery (LC3) through a specific sequence called LC3-interacting region (LIT) motif[9-12]. For example, p62/SQSTM1 (p62) participates in aggrephagy (protein aggregate autophagy) and p62 binds ubiquitinated protein aggregates through an ubiquitin-associated (UBA) domain. On the other hand, BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), which is a mitochondrial localized BH-3 only Bcl-2 family protein, is involved in mitophagy (mitochondrial autophagy). Both p62 and BNIP3 induce degradation of their specific target by autophagy via their LIT motifs[10-13].

AUTOPHAGY AND CANCER

As a pro-survival pathway, the role of autophagy in cancer has long been speculated. However, significant evidence suggests that autophagy might participate in both tumor suppression and tumor maintenance. Furthermore, the resistance to chemotherapy, which is one of the major obstacles in the treatment of cancers, has been linked to autophagy, as supported by the latest studies. This multiplicity function of autophagy in cancer is discussed in detail below.

Autophagy as a tumor suppression mechanism

The first evidence of a tumor suppressive role of autophagy in cancer originated from the observation that heterozygous loss of the Beclin1 encoding gene (*Becn1*) was detected in breast, ovarian and prostate cancer[14]. Subsequent studies with mouse models further established the role of autophagy in tumor suppression. *Becn1* heterozygous knockout mice developed tumors of both benign and malignant nature in various tissues[15,16], suggesting that *Becn1* is a haploinsufficient tumor suppressor gene[16]. Similarly, a mouse model with systemic mosaic deletion of Atg5 and the liver-specific homozygous deletion of Atg7 both developed benign liver adenomas[17]. Vice versa, re-introducing beclin-1 into human breast carcinoma cells decreased both the proliferation *in vitro* and tumorigenesis capacity *in vivo*[14]. Apart from the experimental evidence, the tumor suppressive role of autophagy is also supported by the observation that other tumor suppressor genes, such as ULK3, UV irradiation resistance-associated gene and Bif-1, frequently participate in autophagy signaling[2,18]. On the other hand, the overexpression of oncogenes usually imposes a negative effect on autophagic activity. For instance, PI3K/AKT pathway, which is activated in various cancers, suppresses autophagy through mTOR phosphorylation (*i.e.*, activation)[19]. The up-regulation of anti-apoptotic Bcl-2 proteins in cancer also suppresses autophagy via Beclin1, as described above.

The knowledge regarding the mechanisms underlying the role of autophagy in tumor suppression is still limited. However, an interesting study by Mathew *et al*[20] have reported that the allelic loss of Beclin1 results in increased chromosomal instability. They further showed that the altered regulation of nuclear factor κB, which resulted from p62/SQSTM1 (p62) and reactive oxygen species (ROS) accumulation, is responsible for the damage induced by autophagy deficiency[20].

Senescence is also a potential mechanism by which

autophagy can exert a tumor suppressive role. Senescence is the status of cell cycle arrest with active metabolism[21]. Autophagy has been shown to activate senescence in cultured human lung fibroblast cells[21]. Similarly, autophagy has been suggested to mediate senescence in primary biliary cirrhosis[22]. By inducing senescence in transformed cells, autophagy can induce cell cycle arrest in transformed cells and prevent tumorigenesis.

Another plausible route of tumor suppression is through autophagy-mediated cell death[23]. Although the definition and mechanisms by which autophagy induces cell death is still under debate, several studies strongly support a role for autophagic cell death in tumor suppression. Gurpinar *et al*[24] have shown the involvement of autophagy in cell death induced by the treatment of lung adenocarcinoma cells with sulindac sulfide amide. Interestingly, cell death in this system occurred in the absence of caspase activation[24]. In addition, Lamy *et al*[25] have shown that myeloma cells can avoid cell death by restricting the autophagic activity through the cleavage of autophagic inducer, BCL2-interacting protein BCLAF1, by caspase-10.

Autophagy and tumor maintenance

Interestingly, a role in promoting and maintaining tumors has also been suggested for autophagy regarding cancer development. The conditions which induce autophagy, such as nutrient deprivation, hypoxia and reactive oxygen species, are also present in the tumor microenvironment, especially in tumors with limited blood supply. Yang *et al*[26] have shown that the basal level of autophagy is elevated in pancreatic cancers. Blocking autophagy by chemical inhibitors or RNAi methodology inhibits the tumorigenic potential of the cancer cells, as determined by both *in vitro* and *in vivo* assays[26]. Autophagy inhibition is also correlated with a decrease in oxidative phosphorylation and ATP production. Similar findings were also reported with Ras-transformed immortal, nontumorigenic mouse kidney epithelial cells isolated from baby mice[27]. In addition, the requirement for a functional autophagy machinery for Ras-induced cellular transformation has also been confirmed in other cell models[28,29].

The understanding of the mechanisms by which autophagy supports oncogenic growth is still in its infancy. One possibility is that autophagy process might be used by cancer cells to meet their energy requirements. As discussed in earlier sections, there is a connection between autophagy inhibition and the depletion of intracellular ATP stores and oxidative phosphorylation[26,27]. However, the requirement of oxidative phosphorylation by cancer cells is unclear because cancer cells have been suggested to be dependent more on glycolysis to fuel their growth (aka Warburg effect) even in the presence of oxygen (*i.e.*, aerobic glycolysis)[30]. Nevertheless, some studies point to the intriguing possibility that cancer cells can stimulate autophagy in the adjacent stromal cells, which in turn provide cancer cells with metabolic substrates[31]. Another potential mechanism may be linked to the organelle quality control function of the autophagy process. Damaged organelles, such as mitochondria, can be targeted for autophagy by the BH-3 only Bcl-2 family members including Pink3, BNIP3 and Nix proteins[12,13,32]. Of note, any damage to mitochondria will induce ROS production and may lead to genomic instability[33,34]. Autophagy has also been shown to be directly involved in the degradation and elimination of oxidized proteins. Despite playing a positive role in the initial stages of tumorigenesis, oxidative stress and genomic instability are detrimental to tumor growth in the later stages[2]. It is therefore feasible that autophagy can mitigate these damages and thereby sustain oncogenic growth[35].

Autophagy and tumor therapy resistant tumors

An association between autophagy and the effectiveness of treatment has also been suggested by recent studies. For example, autophagy has been reported to be elevated in pancreatic cancer cells treated with chemotherapeutic drugs[36-38]. It should however be noted that there was no consensus as to whether the increased autophagic activity contributes to cell death[36,37] or facilitates cancer cell survival under stress conditions in pancreatic cancer[38]. On the other hand, in a different model using Myc-induced lymphoma, Amaravadi *et al*[39] have reported that chemotherapy induces autophagy and that the inhibition of autophagy enhances apoptosis induced by chemotherapy drugs. Furthermore, autophagy inhibitors, such as chloroquine and hydroxychloroquine have been shown to exhibit a synergistic effect with chemotherapy and radiotherapy[39-43]. Autophagy inhibitors are currently being tested in clinical trials as part of the combined therapy approach for various cancers[6].

Tumor maintenance function (see above) of autophagy may also alleviate the stress induced by cancer therapy and thereby induce therapy resistance. Besides, the new role ascribed to autophagy in the regulation of cancer stem cell (CSC) phenotype[44,45] might serve as a potential mechanism for autophagy to promote therapy resistance. The so-called "cancer stem cell theory" has generated lively discussion in recent years. CSCs are a small (< 5%) subpopulation of heterogeneous cancer cells, which are capable of self-renewing and differentiating into the whole spectrum of tumor cell population. CSCs have also been suggested to be resistant to treatment[46-50]. Despite the controversy, which still exists regarding the characteristics of CSC in various solid tumors, a correlation between autophagy and CSC population has been suggested. Autophagy has been shown to be involved in the maintenance of CSCs in breast cancer[51]. Accordingly, inhibiting ATG12 and LC-3 by siRNA methodology or with the pharmacological inhibitors of autophagy altered the phenotype of breast CSCs[51]. Similarly, Rausch *et al*[52] reported that the autophagic markers co-localize with CSC markers in tumors which were surgically removed from pancreatic cancer patients. The pancreatic cancer cell line, MIA-PaCa2 has been shown to exhibit more prominent stem-like properties (as determined by

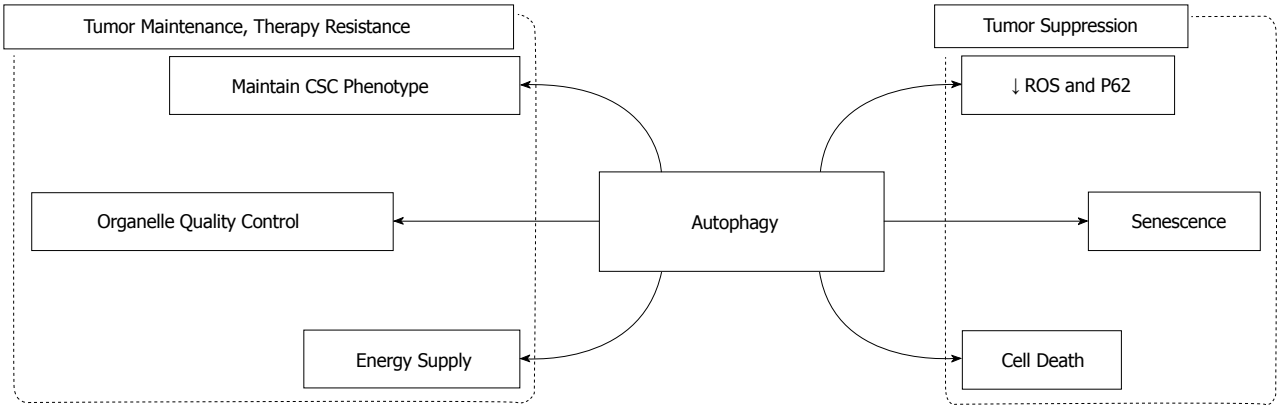

Figure 1 Schematic summary of the role of autophagy in cancer. Autophagy contributes to tumor suppression as well as tumor maintenance and therapy resistance. The mechanisms by which autophagy is involved in tumor suppression include limiting the accumulation of ROS and P62, and inducing senescence and cell death. On the other hand, autophagy facilities tumor maintenance and therapy resistance by providing the tumor with metabolic substrates and maintaining intracellular homeostasis (organelle quality control), and by possibly contributing to the maintenance of CSC phenotype. CSC: Cancer stem cell; ROS: Reactive oxygen species.

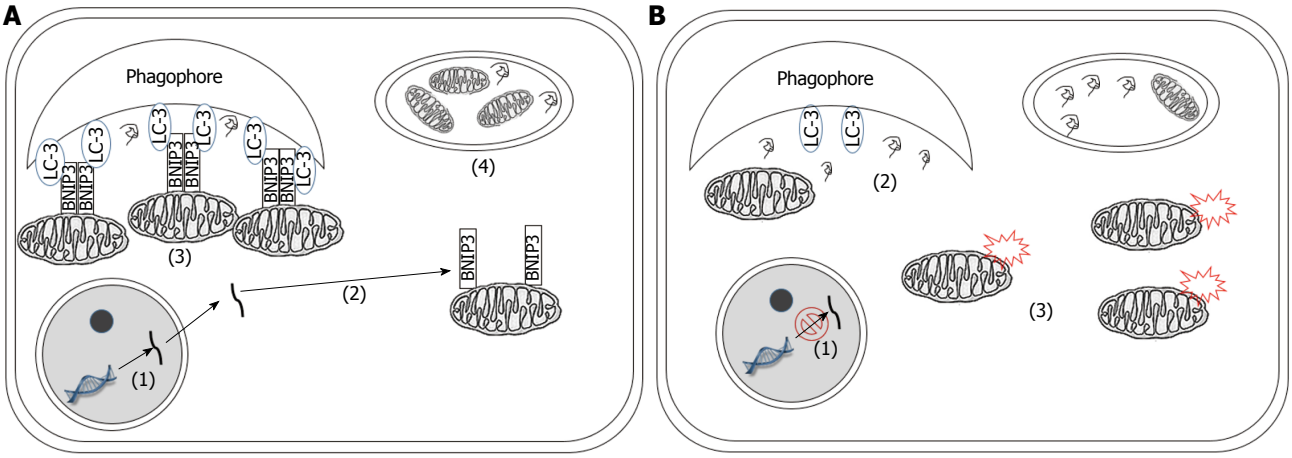

Figure 2 Schematic illustration of differential regulation of mitophagy participating in the progression of pancreatic cancer. Numbers in parentheses indicate successive mitophagy processes that are interrelated. A: During the early stages of pancreatic cancer: (1) BNIP3 is transcribed; (2) translated and inserted into mitochondria membrane; (3) Active BNIP3 tethers mitochondria to the phagophore through its interaction with LC-3;(4) Mitochondria are therefore selectively engulfed in the autophagosome and degraded by the lysosome. In this way, mitochondria-induced ROS production is limited and genome stability is preserved; B: In the later stages of pancreatic cancer: (1) *BNIP3* gene is silenced; (2) The absence of *BNIP3* on the mitochondrial outer membrane will prevent the process of selective targeting of mitochondria to the autophagy machinery; (3) Accumulation of damaged mitochondria will result in elevated production of ROS and increased genome instability, which further contributes to the progression of cancer.

functional assays) compared to another pancreatic cancer cell line, BxPc-3[53]. In accordance with their stronger stem-like features, MIA-PaCa2 cells also displayed higher autophagic activity[54] and resistance to cell death induced by chemotherapeutic drug gemcitabine[54,55] than that observed with BxPc-3 cells. It is therefore possible that autophagy is associated with the maintenance of the stem cell phenotype of pancreatic CSCs and thereby contributes to the resistance observed with therapy. Nevertheless, the underlying mechanisms by which autophagy modulates CSC phenotype and contributes to drug resistance requires further research.

AUTOPHAGY-A DOUBLE-EDGED SWORD

In summary, based on current knowledge, autophagy can act both as a positive and negative regulator of tumor growth in various cancers (Figure 1). Several hypotheses have been proposed to reconcile these seemingly contradictory observations, which can be summarized as follows: (1) The differential effects of autophagy in cancer might be attributed to the tissue specificity. This is supported by the fact that the highest correlation between tumor growth and elevated autophagy is observed in Ras-induced oncogenesis[2]; (2) A dynamic role for autophagy has been proposed in the development of cancer. Namely, autophagy might play a suppressive role in the initiation stages of cancer but support the maintenance of tumor growth in the later stages of tumorigenesis. This hypothesis is supported by the observation that homozygous Atg5 and Atg7 KO mice, which display more significant autophagic inhibition, developed only benign tumors[17]. In contrast, *Becn1* KO mice, which exhibit relatively higher level of autophagy, displayed both benign and malignant tumors[2,15,16]; and (3) The substrate selectivity of autophagy has recently emerged as a poten-

tial mechanism responsible for the differential roles of autophagy in cancer. Mitophagy has been shown to be activated in Ras transformed cells. Autophagy deficiency results in accumulation of abnormal mitochondria when cells are challenged with starvation[27]. In contrast, in pancreatic cancer cells, initial attempts have failed to detect any significant mitophagic activity[26]. Interestingly, the specific receptor for mitophagy, BNIP3, has been found to be silenced in various pancreatic cancer cell lines[56,57]. Since damaged mitochondria are the major source of ROS which promote tumorigenesis and malignant transformation, it is feasible that mitophagy might serve as a protective mechanism in the initial stage of tumorigenesis (Figure 2A). The loss of this protective role, resulting from the silencing of mitophagic receptor, may promote the tumor to a more advanced stage (Figure 2B). Indeed, immunohistochemical staining of BNIP3 in pancreatic tissues indicated that BNIP3 silencing is a late event in pancreatic cancer pathogenesis[56]. In the early stage pancreatic cancer tissues, BNIP3 exhibits a perinuclear distribution pattern[56]. These findings strongly suggest that mitophagy is activated in the early stages of pancreatic cancer[58-60]. In contrast, this distinct pattern of BNIP3 expression is missing in late stages of pancreatic adenocarcinoma. In addition, Takahashi *et al*[61] have found that haploinsufficiency of a tumor suppressor gene, Bif-1, attenuates mitophagy and subsequently promotes chromosomal instability in a mouse model of B-cell lymphoma. Similarly, mitochondrial content has been shown to be elevated in breast cancer[62], colorectal cancer[63], and ovarian cancer[64]. Although direct evidence is still lacking for the substrate specificity of autophagy in cancer, further studies are required to understand the importance of this mechanism in various cancers.

CONCLUSION

Studies so far support both a tumor suppressive and an initiative role for autophagy in cancer. These differential effects of autophagy could be due to several reasons including the tissue specificity of tumors and the different stages of tumorigenesis. The role of substrate specificity of autophagy (*e.g.*, mitophagy) and other potential mechanisms warrant further research. It is of great importance that we improve our understanding of the roles which autophagy plays in cancer. Notably, this will enable the development of individualized treatments for cancer patients according to their cancer type and its progression. In fact, autophagy inhibitors are already being tested in clinical trials and hold promise for combined cancer therapies.

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doi:10.4331/wjbc.v4.i3.71

World J Biol Chem 2013 August 26; 4(3): 71-78
ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Potent trophic activity of spermidine supramolecular complexes in *in vitro* models

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Supported by Grants from the Italian Ministry of Education
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Received: April 30, 2013 Revised: July 3, 2013
Accepted: July 23, 2013
Published online: August 26, 2013

Abstract

AIM: To test the growth-promoting activity of the polyamine spermidine bound to various polymeric compounds in supramolecular complexes.

METHODS: A thiazolyl blue cell viability assay was used to determine the growth-promoting potency of spermidine-supramolecular complexes in a human skin fibroblast cell line exposed to spermidine and different spermidine-supramolecular complexes that were obtained by combining spermidine and polyanionic polymers or cyclodextrin. Reconstituted human vaginal epithelium was exposed to a specific spermidine-supramolecular complex, *i.e.*, spermidine-hyaluronan (HA) 50, and cell proliferation was determined by Ki-67 immunohistochemical detection. Transepithelial electrical resistance and histological analysis were also performed on reconstituted human vaginal epithelium to assess tissue integrity.

RESULTS: The effect of spermidine and spermidine-supramolecular complexes was first tested in skin fibroblasts. Spermidine displayed a reverse dose-related mode of activity with mmol/L growth inhibition, whereas 30% stimulation over basal levels was detected at μmol/L and nmol/L levels. Novel spermidine-supramolecular complexes that formed between spermidine and polyanionic polymers, such as HA, alginate, and polymaleate, were then tested at variable spermidine concentrations and a fixed polymer level (0.1% w/v). Spermidine-supramolecular complexes stimulated the cell growth rate throughout the entire concentration range with maximal potency (up to 80%) at sub-μmol/L levels. Similar results were obtained with spermidine-(α-cyclodextrin), another type of spermidine-supramolecular complex. Moreover, the increased expression of Ki-67 in the reconstituted human vaginal epithelium exposed to spermidine-HA 50 showed that the mode of action behind the spermidine-supramolecular complexes was increased cell proliferation. Functional and morphological assessments of reconstituted human vaginal epithelium integrity did not show significant alterations after exposure to spermidine-HA, thus supporting its safety.

CONCLUSION: Spermidine found in spermidine-supramolecular complexes displayed potentiated regenerative effects. Safety data on reconstituted human vaginal epithelium suggested that assessing spermidine-supramolecular complex efficacy in atrophic disorders is justified.

Key words: Polyamines; Spermidine; Growth; Supramolecular complexes; Vaginal epithelium; Ki-67

Core tip: Previous *in vitro* studies showed that spermidine may have different, or even opposite, effects on cell survival, leading to either proliferation or apoptosis depending on a variety of factors. We showed that the inclusion of spermidine in supramolecular complexes with various polymers optimized its use for regenerative purposes. Spermidine-supramolecular complexes

stimulated cell proliferation but did not cause significant alterations to vaginal tissue integrity. These results suggest that growth pathways in senescent or damaged tissues may be activated by the controlled release of spermidine from spermidine-supramolecular complexes to provide a faster recovery.

Ghisalberti CA, Morisetti A, Bestetti A, Cairo G. Potent trophic activity of spermidine supramolecular complexes in *in vitro* models. *World J Biol Chem* 2013; 4(3): 71-78 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i3/71.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i3.71

INTRODUCTION

Spermidine belongs to the class of biogenic, small, polycationic polyamines found ubiquitously in microbial, plant, and animal cells. These compounds interact with negatively charged molecules such as DNA, RNA or proteins and, thus, are involved in a wide array of functions. It has been shown that polyamines play a role in DNA and RNA stabilization, the modulation of mRNA translation, and cellular signaling[1-3]. Their large range of involvement in processes and cellular responses suggests that polyamines play a key role in the control of cell growth, survival, and proliferation.

In humans, spermidine is derived from three sources: cellular synthesis, food intake, and production by the gut microflora. In normal healthy cells, the levels of spermidine and other polyamines are tightly controlled by multiple mechanisms that balance synthesis, catabolism and transport. The first biosynthetic step is the production of *L*-ornithine from *L*-arginine by mitochondrial arginase. Ornithine is then decarboxylated by ornithine decarboxylase to produce putrescine, which is then transformed into spermidine by spermidine synthase. Spermidine may be further converted to spermine by spermine synthase[4].

Changes in polyamine levels have been associated with aging and disease, with levels declining continuously with age[5]. However, several lines of evidence have indicated the beneficial effects of polyamines; dietary spermidine supplementation showed protective effects against aging in human cells and laboratory animals. Moreover, epidemiological studies showed that prolonged life expectation was associated with higher polyamine food intake[6-11]. Experimental studies showed that polyamines are essential for eukaryotic cell growth and viability, as they were found to positively promote vital functions such as proliferation, cell reprogramming, autophagy, migration and differentiation[12-15].

However, not all of the data obtained in cultured cells are supportive of a positive role for spermidine on cell growth. For example, the role of polyamines in cell death, particularly apoptosis, is still controversial. In different cellular models, polyamine depletion inhibited mitochondrial and death receptor-dependent apoptosis pathways[16]. Conversely, another study indicated that polyamine-depletion in IEC-6 cells delayed the onset of apoptosis and conferred protection against DNA damaging agents, suggesting an involvement of the caspase activating signal cascade[17]. Examples of either activation or prevention of apoptosis due to polyamine depletion are known for several cell lines[18].

The dual activity of polyamines may depend on the fact that the local concentration of spermidine seems to be a critical factor, as was shown for its effect on microtubule assembly[19]. Another important factor is the working mechanism of polyamines, which seem to perform their different functions through the formation of supramolecular complexes with large anionic molecules such as DNA and RNA, as was shown for lymphocytes, in which it is estimated that 60% of spermidine is bound to RNA[20]. The formation of different spermidine-supramolecular complexes that release sustained, minute amounts of polyamines may influence the concentration, and hence the activity, of spermidine, *e.g.*, by triggering reparative processes on senescent or damaged tissues. In view of a possible therapeutic use of spermidine to restore physiological status in tissues undergoing senescence-dependent alterations, finding optimal conditions (*i.e.*, formation of spermidine-supramolecular complexes) for such use is critical. In particular, it is conceivable that spermidine could restore a healthy status in urogenital tissues and mucosae undergoing senescence, a common condition affecting a large segment of postmenopausal women. To investigate this issue, we tested the effects of spermidine and various spermidine-supramolecular complexes in fibroblasts and in reconstituted human vaginal epithelium.

MATERIALS AND METHODS

Chemicals

Spermidine 3HCl (Sigma-Aldrich, Milan Italy); Hyaluronate high molecular weight, sodium salt (Bioiberica, Barcelona, Spain); Oligomeric hyaluronate (Tixupharma, Milan, Italy); Sodium alginate (Santialgine™ S1100, Cargill, United States); co-[methylvinylether/maleic acid]-polymer, sodium salt, (PMVE/MA, Gantrez™ S-97 BF, ISP, United States); α-Cyclodextrin (Cavamax® W6 Pharma, Wacker Chemie AG).

Preparation of Spermidine supramolecular complexes

Spermidine-supramolecular complexes with polyanionic polymers were prepared by adding 0.1% w/v of the following polymers to a 1/10 serially diluted solution of spermidine: hyaluronate (HA), oligomeric hyaluronate (OHA), sodium alginate (Alg), and gantrez (Gz). The resulting spermidine-supramolecular complexes are illustrated in Table 1, expressed as the ratio (meq/meq) of cationic equivalents from spermidine and anionic equivalents from the anionic polymer. For the assay on reconstituted human vaginal epithelium tissue, a spermidine-supramolecular complex containing spermidine

Table 1 Spermidine-supramolecular complexes tested in the fibroblasts proliferation model

Spermidine concentration	Spermidine-HA	Spermidine-Alg	Spermidine-Gz
0 nmol/L	HA 0.1%	Alg 0.1%	Gz 0.1%
1 nmol/L	SMC 10^{-6}:1	SMC 10^{-6}:3	SMC 10^{-6}:9
10 nmol/L	SMC 10^{-5}:1	SMC 10^{-5}:3	SMC 10^{-5}:9
100 nmol/L	SMC 10^{-4}:1	SMC 10^{-4}:3	SMC 10^{-4}:9
1 μmol/L	SMC 10^{-3}:1	SMC 10^{-3}:3	SMC 10^{-3}:9
10 μmol/L	SMC 10^{-2}:1	SMC 10^{-2}:3	SMC 10^{-2}:9
100 μmol/L	SMC 10^{-1}:1	SMC 10^{-1}:3	SMC 10^{-1}:9
1 mmol/L	SMC 1:1	SMC 1:3	SMC 1:9

Each spermidine-supramolecular complex is represented as spermidine-to-polymer ratio (meq/meq), as calculated from the cationic equivalents from spermidine and the anionic equivalents from the polymer. HA: Hyaluronate; OHA: Oligomeric hyaluronate; Alg: Sodium alginate; Gz: Gantrez; SMC: Spermidine-supramolecular complexes.

(5 μmol/L) and HA in a 1:50 meq:meq ratio, hereafter termed spermidine-HA50, was prepared.

The spermidine-supramolecular complex as an inclusion complex was prepared by dissolving 1.944 g of α-cyclodextrin (aCD) in 15 mL of water; this solution was added to 1 mmol of spermidine 3HCl, and then 0.2 mL of 1 mol/L NaOH was added under slow stirring. This stock solution was serially diluted to reach the required concentrations.

Cell culture experiments

The human skin fibroblast cell line ATCC-CRL-2703 was grown under standard culture conditions in DMEM. Spermidine and the different spermidine-supramolecular complexes were added to wells containing semi-confluent cells. Cells were exposed to test products for 24 and 48 h (medium was replaced after 24 h). At the end of the incubation period, cells were washed with 200 μL of PBS, and cell viability was evaluated using thiazolyl blue (MTT) as an indicator of mitochondrial function. Briefly, 200 μL of MTT-medium was added to each culture well and left for 4 h at 37 ℃ and 5% CO_2. The MTT-medium was then removed, and 200 μL of MTT solvent solution (10% Triton X-100 plus 0.1 mol/L HCl in anhydrous isopropanol) was added. The plate was shaken on a rotary plate for 20-30 min to ensure that all of the formazan crystals were dissolved. Absorbance was measured at 570 nm on a microplate reader, and the background absorbance at 690 nm was subtracted. For each concentration, six replicates were carried out. The results are expressed as percentage (%) cell proliferation compared to an untreated control cell culture.

Ki-67 immunohistochemistry in reconstituted human vaginal epithelium

Reconstituted human vaginal epithelium, obtained from SkinEthic™ (Lyon, France), was derived from the immortalized human cell line A431 grown for 5 d on polycarbonate filters. Reconstituted human vaginal epithelium has a strict morphological similarity to the normal vaginal epithelium. Reconstituted human vaginal epithelia were placed in 6- or 12-well plates containing 0.75-1.00 mL of maintenance medium and incubated at 37 ℃ in 5% CO_2 overnight. The reconstituted human vaginal epithelia (duplicate samples per treatment) were incubated for 24 h at 37 ℃ with 50 μL of a 1 mg/mL aqueous solution of spermidine-HA 50 and then fixed in 10% formol-saline for immunohistochemical processing. To evaluate the expression of Ki-67, a known marker of cell proliferation, the Ki-67 antibody (Invitrogen, Eugene, OR, United States) was applied overnight at a 1:100 dilution, and detection was obtained using a commercial kit (Superpicture Polymer Detection Kit, Invitrogen) containing horseradish peroxidase and diaminobenzidine (DAB) as chromogenic agents.

Transepithelial electrical resistance and histological evaluation of reconstituted human vaginal epithelium

Transepithelial electrical resistance, which is the discriminating parameter in the EU-validated rat skin electrical resistance test to measure the integrity of stratum corneum and its barrier function, was evaluated in reconstituted human vaginal epithelia. The higher the transepithelial electrical resistance value, the lower the loss of intercellular water content. Transepithelial electrical resistance of reconstituted human vaginal epithelia was measured in triplicate before and after exposure to spermidine-HA50, prepared as described above, or to physiological saline for 24 h at 37 ℃. Briefly, 5 mL of spermidine-HA50 or physiological saline was applied to the tissue sample in a plate containing 5 mL of physiological saline; transepithelial electrical resistance was evaluated in the range of 0-20 kΩ relative to the tissue surface by a Millicell ERS instrument (Millipore, MA, United States). Three measurements were made before and after treatment; the latter measurement was performed after washout of the compound. The value (Ω) of the void support was subtracted from the values obtained for tissues; a further correction was made regarding tissue surface (0.5 cm^2) as follows: mean value (Ω) of 3 determinations – value of void support = value (Ω) × 0.5 (tissue surface). At the end of the procedure, the tissue was rinsed and fixed in 10% formol saline, processed through paraffin wax, cut and stained with hematoxylin-eosin for histopathological examination (× 20 magnification, DFC-320 Leica camera). Tissue damage was scored from 0 to 4, in order of increasing severity, by a blinded examiner.

Statistical analysis

The data were subjected to statistical analysis using a *t*-test for independent samples. For spermidine and spermidine+cyclodextrin groups, each experimental time point was compared to untreated cells, and the results were considered significant for $P < 0.05$. For the spermidine + HA, spermidine + OHA, spermidine + Alg and spermidine + Gz groups, each experimental time point was compared to cells treated with the respective vehicle (HA, OHA, Alg, Gz), and the results were considered significant for $P < 0.05$.

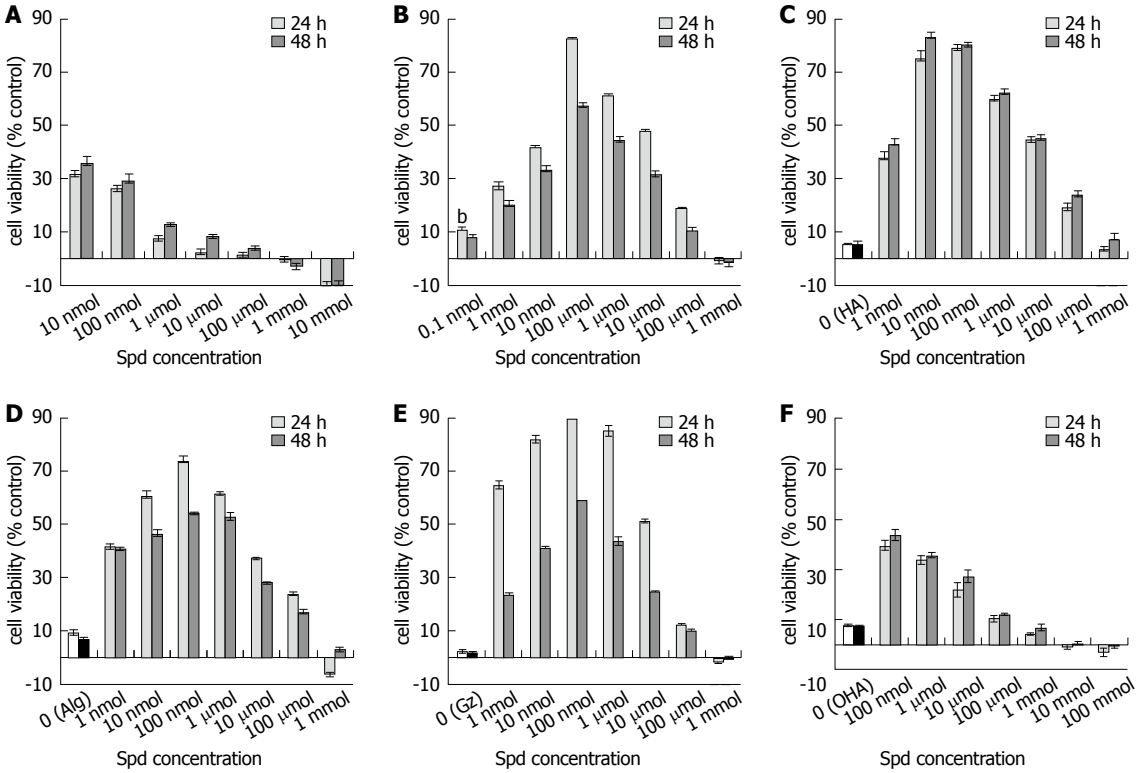

Figure 1 Effect of spermidine and spermidine-supramolecular complexes on cell growth. Fibroblast cultures were exposed to spermidine salts (A) or spermidine-supramolecular complexes (B-F) for 24 (dark bars) and 48 h (light bars) and cell viability was evaluated by thiazolyl blue assay. A: Cells were treated with pure spermidine 3HCl (spermidine); B: Supramolecular complexes formed by spermidine with α-cyclodextrin (spermidine-aCD); C: Polymeric hyaluronan (spermidine-hyaluronate, HA); D: The natural biopolymer alginate (Spermidine-sodium alginate, Alg); E: Synthetic maleate copolymer (gantrez, Gz); F: Depolimerized HA salt (oligomeric hyaluronate, OHA). These data (mean ± SE, *n* = 3) are expressed as percentage (%) increase over cells treated with physiological saline (panels A and B) or the supramolecular complex alone without spermidine (panels C-F).

RESULTS

Spermidine-based supramolecular complexes stimulated cell growth

To evaluate the effect of spermidine on cell growth, human fibroblasts were exposed to spermidine either as a free salt or in various spermidine-supramolecular complexes (Table 1). The exposure of fibroblasts to spermidine alone for 24 or 48 h increased the cell number compared to untreated cells by approximately 30% at 10 and 100 nmol/L. However, higher concentrations resulted in the progressive reduction of the stimulatory effect, with the highest doses showing an almost linear, dose-dependent inhibitory effect (Figure 1A).

Conversely, the inclusion of spermidine in spermidine-supramolecular complexes produced a completely different bell-shaped response pattern (Figure 1B-E). Spermidine in complex with α-cyclodextrin (Spermidine-aCD) that was serially diluted to provide concentrations ranging from 1 to 0.1 nmol/L exhibited excellent stimulatory activity, with a peak at 100 nmol/L showing 80% stimulation over untreated cells and a decrease in stimulation at higher concentrations (Figure 1B).

Exposure to spermidine-supramolecular complexes formed with different polyanionic polymers such as HA and Alg also resulted in positive responses. Because Alg and HA themselves may display regenerative properties, the effect of spermidine-supramolecular complexes was compared to that of the polymers alone. Spermidine-HA increased fibroblast cell viability by approximately 80% within the 10-100 nmol/L range (Figure 1C). Spermidine-Alg produced a similar, although flatter, bell-shaped curve (Figure 1D). Maximal activity was observed at 10 to 1 μmol/L. It is noteworthy that Alg alone showed better stimulatory properties than HA, a well-known and widely applied regenerative biopolymer. Spermidine-Gz, a complex between spermidine and a synthetic polymer, also showed a strong effect; interestingly, spermidine-Gz activity at 24 h was roughly two-fold higher than at 48 h (Figure 1E). The rich anionic density of the polymethylvinylether-co-maleic anhydride, polymer that constitutes Gz and an expected slower release of entrapped spermidine may account for the higher effect of spermidine-Gz at 24 h. Importantly, exposure of fibroblasts to OHA, a salt formed by depolymerized HA and spermidine, showed a pattern similar to that obtained with spermidine (compare Figure 1A and F), *i.e.*, greater stimulation than depolymerized HA alone at low concentrations, but no effect at doses above 100 μmol/L. This reinforces the idea that the association of spermidine with polymers in supramolecular complexes provides a better stimulating effect over a wide range of concentrations. To better extrapolate the effect of the association between spermidine and spermidine-supramolecular complexes, the

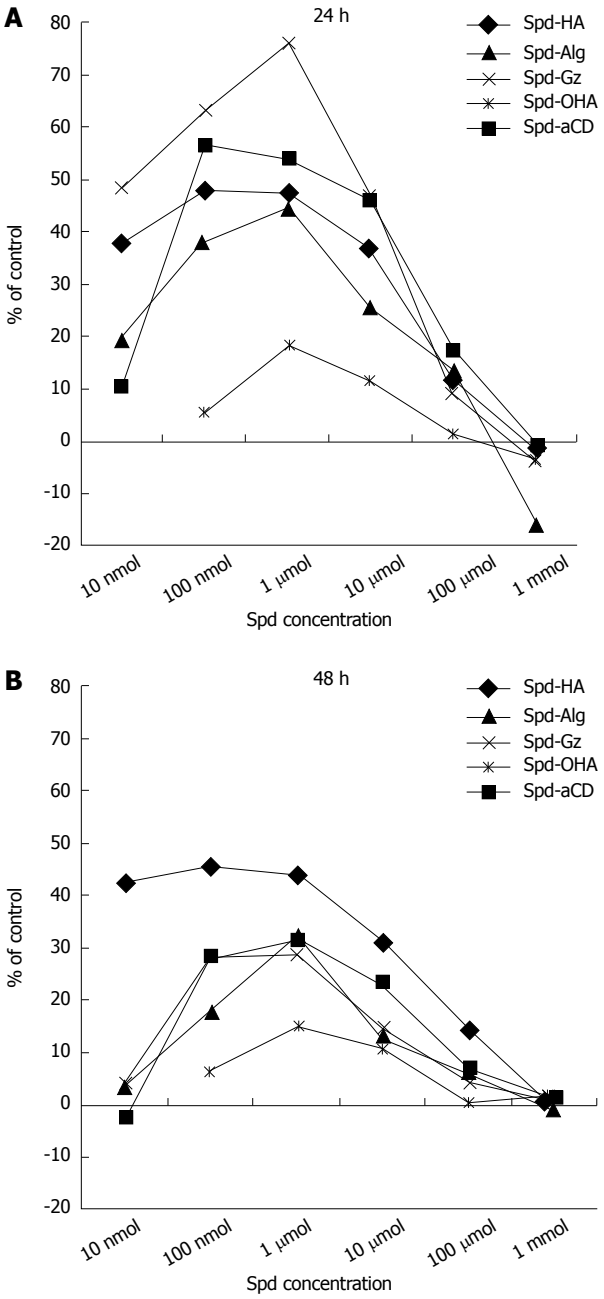

Figure 2 Net proliferative effect of spermidine-supramolecularcomplexes. Data expressed as value of the increment in percentage (%) fibroblasts increase carried out by the spermidine-supramolecular complexes subtracted from the values of spermidine alone at equivalent concentrations and the value of the supramolecular complex-forming polymers at 0.1% concentration. HA: Hyaluronate; Alg: Sodium alginate; Gz: Gantrez; OHA: Oligomeric hyaluronate; aCD: α-cyclodextrin.

data were processed by subtracting the contribution of both spermidine at corresponding concentrations and the relative polyanion for each spermidine-supramolecular complex. The results reported in Figure 2 illustrate the efficacy profile of the association by itself.

Spermidine-supramolecular complexes stimulate cell proliferation in reconstituted human vaginal epithelium

We next investigated the molecular pathways behind the growth stimulating effect of spermidine-supramolecular

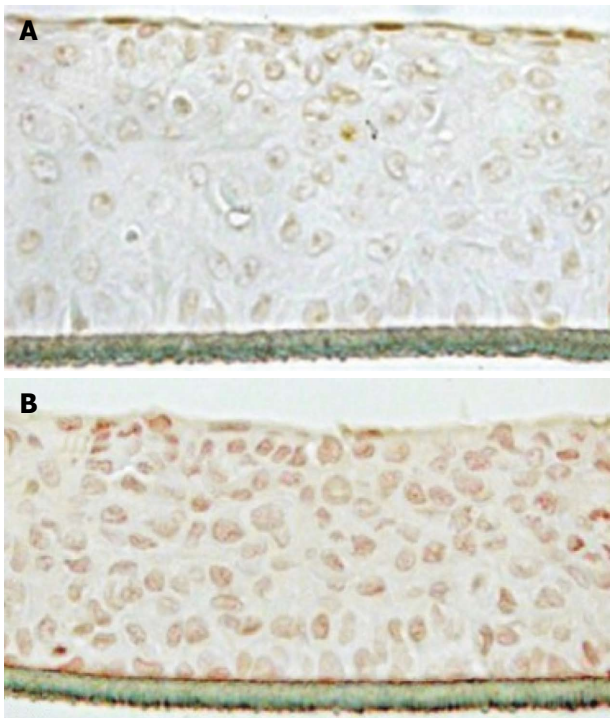

Figure 3 Evaluation of Ki-67 antigen expression in reconstituted human vaginal epithelium. Ki-67 immunoreactivity was evaluated in reconstituted human vaginal epithelium exposed for 24 h to saline (A) or to spermidine-hyaluronate 50 (B). A typical result out of three independent experiments is shown (× 20 magnification).

complexes. To this purpose, the spermidine-HA complex was selected due to its superior balance between regenerative potency and biocompatibility. In fact, spermidine-HA was not inhibitory at any tested concentration (Figure 1C). The spermidine-supramolecular complex formed with a cationic to anionic ratio of 50:1 meq/meq, herein termed spermidine-HA 50, was evaluated in reconstituted human vaginal epithelium by examining the immunohistochemical expression of the mitotic marker Ki-67. In control samples, Ki-67, which is a nuclear protein associated with ribosomal RNA transcription and cell growth and is expressed in the G_1, S, G_2 and mitosis phases of the cell cycle[21,22], was observed only in a few apical cells (Figure 3A). However, many cells expressing Ki-67 homogeneously distributed from the apical to the basal stratum were found in samples exposed to spermidine-HA 50 (Figure 3B). The tissue had a normal cell morphology and extracellular matrix structure and adhered normally to support.

Spermidine-supramolecular complexes are not harmful in reconstituted human vaginal epithelium

The transepithelial electrical resistance assay, which directly measures total resistivity due to the combined thickness and function of cell tight junctions, evaluates the degree of damage and functionality of tissues. We thus used transepithelial electrical resistance in reconstituted human vaginal epithelium to check for possible damaging effects of spermidine-HA 50. Figure 4A shows that after 24 h of treatment, the resistivity of control samples increased by about 10%, whereas that of

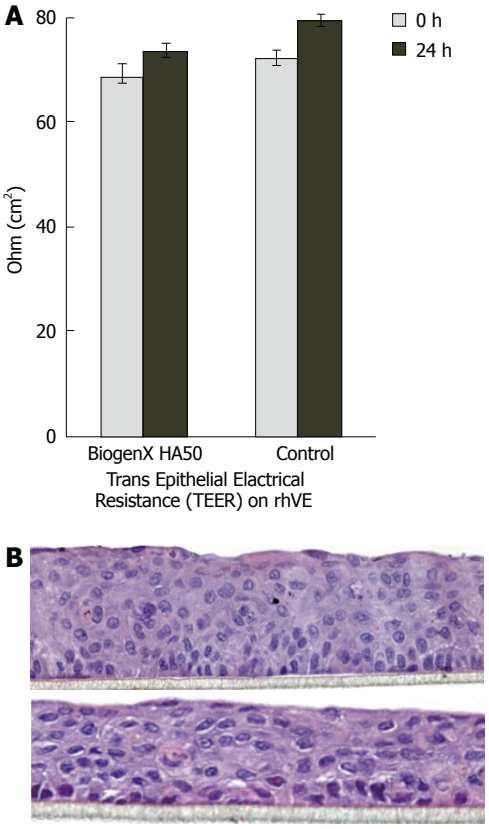

Figure 4 Histopathological examination and transepithelial electric resistance assessment of reconstituted human vaginal epithelium. A: Cell integrity was evaluated by transepithelial electrical resistance determination (Ω/cm^2). Mean values of separate 3 mean ± SE; B: Reconstituted human vaginal epithelia were examined histologically after exposure to spermidine-hyaluronate 50 (upper panel) or saline (lower panel). A typical result out of three independent experiments is shown (× 20 magnification).

the spermidine-supramolecular complex-treated samples increased by 7%. The values fell in the normal range of 60-80 Ω/cm^2, and the difference, though not statistically significant, was regarded as a fluctuation without pathological significance. Baseline values accounted for less than 5% of the difference.

Histological examination did not show tissue alterations (Figure 4B). In samples exposed to spermidine-HA 50 for 24 h (upper panel), the tissue condition, cell morphology, adherence to support, appearance of the extracellular matrix, and continuity to the squamous external layer appeared essentially unchanged with respect to reconstituted human vaginal epithelium treated for 24 h with saline (lower panel).

DISCUSSION

In vivo, polyamine metabolism is tightly regulated by complex machinery involving biosynthesis, interconversion, catabolism, and cellular uptake to reach the appropriate levels of these regulatory factors[23]. Due to their cationic nature, polyamines, including spermidine, strongly bind to DNA, thereby changing its conformation. Moreover, spermidine, by modulating histone acetylase and deacetylase activity, can alter histone acetylation, which in turn will affect gene transcription in proliferating cells[24] that contain the amino acid residue hypusine, which is specifically synthesized from spermidine. Therefore, by modulating gene expression at multiple levels, polyamines (and spermidine in particular) trigger a variety of changes that can potentially lead to complex cellular responses[13,25]. Such a variety of different actions, together with the requirement for appropriate tissue concentrations, may explain how, in experimental studies, polyamines were able to promote both cell growth and cell death and showed complex involvement in various pathophysiological conditions[26]. This finding suggests that the proper concentrations are required to attain a high positive response for regenerative purposes.

In our skin fibroblast model, spermidine showed an expected growth-promoting effect, but polyanionic-type supramolecular complexes containing spermidine were overall much more effective in increasing cell viability, with an effect generally more pronounced at 24 h than at 48 h (Figure 2). Moreover, while the stimulating effect of spermidine alone on cell viability was evident only at low concentrations, time course and dose response studies showed that spermidine-supramolecular complexes promoted cell growth over a wide range of concentrations without inhibitory effects. The trends depicted bell-shaped curves with peaks exceeding an 80% increase over basal levels and showed different profiles (Figure 1). The strongest effect on cell viability was obtained by the association with a typical growth-enhancing biopolymer, such as HA, that resulted in a potent synergistic effect. The cumulated potency of spermidine-HA was 15-fold higher than that of HA alone, and it did not show the bimodal pattern of spermidine alone. Our interpretation of these data suggest that the effect of spermidine-supramolecular complexes might correlate with the kinetics of spermidine release in the culture medium; spermidine-supramolecular complexes may continuously release low amounts of spermidine that simulate the growth-stimulating effect of low doses of spermidine alone (Figure 1A) while avoiding the inhibitory effect of high doses.

Support for this hypothesis is provided by comparing the results obtained with spermidine complexed to polymeric HA to those obtained with a salt formed by spermidine and OHA, *i.e.*, monomers or dimers of glucuronic acid/*N*-acetylglucosamine disaccharide. OHA showed a response comparable to that of spermidine, further indicating that spermidine needs to be complexed with either natural or synthetic polymers in spermidine-supramolecular complexes to effectively promote cell growth.

Further confirmation that the inclusion of spermidine in spermidine-supramolecular complexes may be a key factor in optimizing the growth-promoting properties of spermidine was provided by the results obtained with spermidine-aCD, *i.e.*, a spermidine-supramolecular complex based on an uncharged type of inclusion complex. When serially diluted over a concentration range spanning from 1 to 0.1 nmol/L, these complexes also yielded excellent results that were comparable to those of the spermidine-supramolecular complexes formed with the

polyanionic polymers.

The data obtained in cultured cells were supported by experiments performed in reconstituted human vaginal epithelium that was used as a validated model of human vaginal mucosa[27-30]. To explore the mechanism of action of spermidine-supramolecular complexes, reconstituted human vaginal epithelium was exposed to spermidine-HA, the complex of spermidine with HA that showed the best stimulation in fibroblasts. Upon treatment, the expression of the Ki-67 antigen, which is a nuclear protein associated with cell growth expressed in proliferating cells but absent in quiescent cells[21,22], was detected in many cells from the apical layer to the basal cells. Therefore, these results indicate that spermidine-supramolecular complexes provide a steady, mitogenic amount of spermidine (in the nmol/L range) released, thus eliciting a proliferative effect on epithelial cells and sub-seeding fibroblasts. These data indicated that spermidine-supramolecular complexes are effective in triggering regeneration by inducing cell proliferation, although other effects relying on the inhibition of cell death cannot be ruled out. Our results are in agreement with literature data showing that polyamines are essential factors in tissue remodeling[3,6,31-34].

Finally, because these results suggest that spermidine-supramolecular complexes may be used in restoring a healthy tissue condition in various age-related disorders characterized by a compromised trophic status of mucosae, *e.g.*, in urogenital pathology, safety evidence is required. Transepithelial electrical resistance experiments using reconstituted human vaginal epithelium, which were performed to assess functional and morphological characteristics of the vaginal epithelium after exposure to spermidine-supramolecular complexes, showed only minor alterations after 24 h exposure, indicating that the spermidine-supramolecular complex tolerability is satisfactory.

In conclusion, our *in vitro* results indicate that spermidine-supramolecular complexes may elicit soft-tissue remodeling, and the complex potency seems to be linked to a very low, steady flux of spermidine. The broad range of stimulating activity that spanned 6 logs of concentration seems to indicate the presence of a non-receptorial mechanism leading to cell proliferation.

COMMENTS

Background

Spermidine is a master regulator of the cell cycle with a key role in tissue homeostasis, reparation and proliferation. In the cell, spermidine exerts its effects by interacting with polyanionic polymers and chlatrating moieties to form supramolecular complexes. However, its multifarious functions may lead to mitotic activity or to an opposite, pro-apoptotic pathway. In this respect, the local spermidine concentration seems to be the driving force, which could be modulated in pursuit of regenerative therapies.

Research frontiers

Senescence conditions in women, such as vaginal atrophy and stress urinary incontinence, are a largely unmet medical need occurring in post-menopausal and elderly women or after pregnancy and labor. Both require an effective, more appropriate, and less hazardous therapy than estrogenic treatment. Biogenic polyamines play a key role in tissue homeostasis, but due to their bimodal effects on cell viability and tissue growth, it is of the utmost importance to find appropriate conditions for attaining a positive and strong response for regenerative purposes.

Innovations and breakthroughs

Innovation entails demonstrating that the inclusion of spermidine in supramolecular complexes optimizes its trophic activity. Safety and efficacy evidence supports the assessment of the regenerative properties of spermidine-supramolecular complexes in damaged or senescent tissues.

Applications

These results represent a promising basis for *in vivo* investigations into whether spermidine-supramolecular complexes are able to restore a healthy condition in disorders characterized by the overall senescence or damage of soft tissues or in age-related diseases in general. Women's urogenital diseases, such as vaginal atrophy and stress urinary incontinence, may represent potential applications.

Terminology

Spermidine belongs to the family of biogenic amines, *i.e.*, polyamines, which include its metabolic precursor putrescine and the higher homolog spermine. Polyamines are organic polycations that interact with polyanionic polymers by multiple acid-base interactions. The minimal steric hindrance and the low molecular weight of polyamines also allow for the formation of inclusion complexes with cyclodextrins. Authors studied both types of supramolecular complexes at broad concentration ranges *in vitro*. The thiazolyl blue assay measured the effect of the tested substances on cell viability as a function of mitochondrial function. Hence, the expressions "growth promoting activity", "proliferative effects", "cell proliferation", "stimulatory activity", "stimulatory effect", and "regenerative properties", among others, are used synonymously, *i.e.*, to encompass different possibilities of increased cell viability, including proliferation through mitosis, reduced apoptosis and prolonged life-span by autophagy.

Peer review

This is an interesting study in which the authors analyzed the effect of various spermidine-supramolecular complexes on fibroblast and vaginal epithelium proliferation. The results are sound and suggest that spermidine-supramolecular complexes are potential therapeutic substances that could be used for treating tissue senescence associated with urogenital pathological conditions.

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P- Reviewer Ariga K **S- Editor** Gou SX **L- Editor** A **E- Editor** Yan JL

ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2013 November 26; 4(4): 79-147

Volume End

Baishideng Publishing Group
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WJBC

World Journal of Biological Chemistry

Contents

Quarterly Volume 4 Number 4 November 26, 2013

AIM AND SCOPE

World Journal of Biological Chemistry (*World J Biol Chem*, *WJBC*, online ISSN 1949-8454, DOI: 10.4331), is a peer-reviewed open access (OA) academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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INDEXING/ABSTRACTING

World Journal of Biological Chemistry is now indexed in PubMed Central, PubMed, Digital Object Identifier, and Directory of Open Access Journals.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Quarterly

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Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road, Wan Chai,
Hong Kong, China
Telephone: +852-6555-7188
Fax: +852-3177-9906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLICATION DATE
November 26, 2013

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1949-8454/g_info_20100316155305.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.4331/wjbc.v4.i4.79

World J Biol Chem 2013 November 26; 4(4): 79-90
ISSN 1949-8454 (online)

REVIEW

Rethinking quasispecies theory: From fittest type to cooperative consortia

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Received: June 29, 2013 Revised: August 20, 2013
Accepted: September 3, 2013
Published online: November 26, 2013

Abstract

Recent investigations surprisingly indicate that single RNA "stem-loops" operate solely by chemical laws that act without selective forces, and in contrast, self-ligated consortia of RNA stem-loops operate by biological selection. To understand consortial RNA selection, the concept of single quasi-species and its mutant spectra as drivers of RNA variation and evolution is rethought here. Instead, we evaluate the current RNA world scenario in which consortia of cooperating RNA stem-loops (not individuals) are the basic players. We thus redefine quasispecies as RNA quasispecies consortia (qs-c) and argue that it has essential behavioral motifs that are relevant to the inherent variation, evolution and diversity in biology. We propose that qs-c is an especially innovative force. We apply qs-c thinking to RNA stem-loops and evaluate how it yields altered bulges and loops in the stem-loop regions, not as errors, but as a natural capability to generate diversity. This basic competence-not error-opens a variety of combinatorial possibilities which may alter and create new biological interactions, identities and newly emerged self identity (immunity) functions. Thus RNA stem-loops typically operate as cooperative modules, like members of social groups. From such qs-c of stem-loop groups we can trace a variety of RNA secondary structures such as ribozymes, viroids, viruses, mobile genetic elements as abundant infection derived agents that provide the stem-loop societies of small and long non-coding RNAs.

Key words: Quasispecies; RNA stem-loops; Infectious agents; Cooperative interactions; Evolution

Core tip: Single RNA stem-loops operate solely by chemical laws that act without selective forces, and in contrast, self-ligated consortia of RNA stem-loops operates by biological selection. To understand consortial RNA selection, the concept of single quasi-species and its mutant spectra as drivers of RNA variation and evolution is rethought here. Instead, we evaluate the current RNA world scenario in which consortia of cooperating RNA stem-loops (not individuals) are the basic players. We thus redefine quasispecies as RNA quasispecies consortia and argue that it has essential behavioral motifs that are relevant to the inherent variation, evolution and diversity in biology.

Villarreal LP, Witzany G. Rethinking quasispecies theory: From fittest type to cooperative consortia. *World J Biol Chem* 2013; 4(4): 79-90 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i4/79.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i4.79

INTRODUCTION

Our mainstream picture of RNA virus evolution is determined by the quasispecies concept of Manfred Eigen. It was a very helpful theoretical model within the framework of physical chemistry to paint a picture of

viruses with an evolution rate a multitude faster than evolution of cellular organisms. The core assumptions of quasispecies concept[1-3] are rather clear: (1) there is little knowledge about the origin of viruses or their role in the evolution of the biosphere. Viruses are selfish genetic elements that likely originated out of host organisms (escape theory); (2) viruses represent molecules that generate "information" through mutagenesis, *i.e.*, replication-errors; (3) equations of mass action law govern chemical equilibrium in closed chemical systems. Percentage of components within this is determined by these equations; (4) in an environment of high mutation rate and thermodynamic conditions far from equilibrium, self-replicating entities reach maximum reproductive fitness; (5) the self replication entity is not a single molecule but a "cloud" that consists of variant reproductive molecules; (6) the distribution of these "clouds" in systems far from equilibrium depends on master fittest type and mutant spectra, *i.e.*, dominant replicators, mutants closely related, and not closely related. Each of its sequence-syntax occupies a unique position in the sequence space; and (7) because the genetic code is a real language-not just a metaphor-information- and system-theoretical assumptions based on mathematical concepts of language therefore are the appropriate tools to explain quasispecies evolutionary dynamics.

If we look at the current empirical data, these assumptions do not explain many interactional behaviors and broad influences that have been observed in viral studies. In addition, the following issues are relevant: (1) viruses play large and essential roles in the biosphere evolution of host[4-7]. Phylogenetic analyses and comparative genomics suggest that viruses are older than cellular life[8-10]; (2) most viruses are symbiotic or even endosymbiotic inhabitants of cellular host organisms that can promote adaptability for the host[11-15]; (3) information representing natural codes is not the result of errors in replication processes. All empirical data indicate that natural codes depend on consortia of living agents that generate and use codes for information storage and interactional signalling. Natural codes are essential prerequisites of everyday social interactions. If errors in natural code use occur, this does not enrich information but nearly all empirical data indicate information damage, deformation and decreased informational content. Today it seems rather curious to ground evolution of complexity on error and damage[16-19]; and (4) coherent with Ludwig Wittgensteins assumptions, the meaning of natural code sequences is its use (functional activity), *i.e.*, depends on the context, not on its syntax[20]. Accordingly the meaning (functional activity) of syntactically identical viral nucleic acid sequences varies according to context such as epigenetic imprinting[21-23].

These observations have serious consequences for the "every variant" concept of Eigen's quasi-species that include: (1) biological information of nucleic acid sequences does not occupy a unique position in sequence space but depends on contextual use; (2) because of its context-dependency biological information cannot be sufficiently described by information theory or similar mathematically based concepts of language; (3) (Evolutionary) algorithm-based machines cannot provide contextual real life simulations; (4) sequence space of real life nucleotide sequences is not the result of random assemblies; and (5) viral cloud building in natural habitats occurs different in comparison to abiotic molecule assembly.

Additionally quasispecies that cooperate can have various behavioural motifs not present in a pure physical chemistry. They can (1) compete with and exclude related populations; (2) have minority populations that are crucial for overall fitness[24,25]; (3) can display heterogeneity important for fitness that is not observed in the consensus type[26]; (4) can suppress their own replication through lethal defection[27]; and (5) can be composed of members that can complement and interfere with replication of the collective and many of these features can be observed in clinical infections such as humans with hepatitis C virus[28].

Therefore if quasispecies evolve with the above characteristics and are thus different from prior mathematic models, what then is an appropriate description that is also coherent with the abiotic/biotic split in animated nature? If species is the appropriate term in biology to describe essential common features of related groups of cellular organisms, then quasispecies remains an appropriate term to describe related groups of subcellular agents that play essential roles in evolution of the biosphere.

To denote the above crucial differences to the 20th century concept with its paradigmatic core of master copy (fittest type) and mutant spectra (variants) we propose the concept of quasispecies consortia (qs-c) in which behavioural motifs of cooperative RNA-agents are at the foundation of basic capabilities generating sequence space of *de novo* nucleotide sequences and for inserting, changing or deleting such sequence into host sequences.

FROM MOLECULAR ERRORS TO INTERACTIONAL MOTIFS: RNA PARASITES AS OPEN SPACE INVADERS

In most origin of life scenarios, RNA parasites are considered major barriers for the origin of code and life (systems) which compels these proposals to close off the action of parasites into self contained code systems. But RNA parasites can also provide new and highly dynamic code that is added to the system. Naturally evolved RNA sequences can never be completely specified (or closed), since they must interact with their environment, replicate and undergo adaptation while retaining code that can always be further parasitized. Open systems can thus embrace the capacity of parasites to add novelty. This contrasts sharply with closed systems which must limit all such parasites.

This open feature renders the ability to absolutely specify membership (absolute immunity) as basically

indefinable. Any naturally evolved nucleotide sequence can never be fully secure from as yet undefined parasite agents. But a crucial inference out of this "insecurity" is that parasites provide the inherent capacity for novelty, *i.e.*, the precondition for greater complexity. Since parasites are competent in code, they are not "mistakes". This means that the accepted core requirement of biological innovation, variation, is not met by the explanation model of random mutation (error) and its selection. Instead, RNA parasites provide continued infection and colonization which result in added identity of new RNA to existing RNA groups and thus alter self identity (immunity). These RNAs are thus acting as competent agents-not mutations/errors-which seek to impose new and competent code (identity) onto the system. Such RNA's, however, must interact to attain a stable colonization by inhibiting run-away parasite replication.

The core issue is thus to specify how RNA "agents" emerge from chemicals (ribozymes) to form the needed identity (such as for replicators) and also to form RNA groups that can support themselves and learn new membership.

For all extant life, these agents must have initially been RNA stem-loops. Single RNA stem-loop generation occurs by physical chemical properties solely as demonstrated by natural and randomized RNA experiments[29-31]. If numbers of stem-loops are able to build complex consortia with greater competence, they would then represent the initial cooperative interactions needed to develop living systems that are not present in a strictly chemical world. The resulting system must function to maintain itself. In consortia, the emergence of identity (ability to differentiate self *vs* non-self) is a crucial initial step. Thus we seek to understand how single RNA stem-loop RNAs can become competent RNA consortia. And in so doing, we invoke the central action of RNA parasites and follow how "parasite-derived" RNA stem-loops interact in social collectives promoting innovation, infection, immunity and complex multiple (group) identity[32].

CORRECTIVE AND COLLECTIVE POWER OF VIRUS *VIA* QS-C

The term quasispecies originated from models that described related viral RNA populations resulting from error based variation of the master fittest type[1-3]. It was not initially applied to consortia that showed cooperation. In the ensuing several decades, many laboratory observations were made that indicated more complex collective behaviours for viral quasispecies than were predicted by Eigen's quasispecies equations. Two of the more active laboratories were those of John Holland and Esteban Domingo[33]. The most recent compilation of these studies outlines many of the collective behaviours that have been made with quasispecies[34]. The culmination study that most clearly reported that quasispecies have more complex collective behaviours seems to be the study from the Andino group of poliovirus pathogenesis in a mouse model in which diversity and cooperation were key to viral fitness[35,36]. Such studies led to the set of statements above on the cooperative nature of quasispecies. Thus quasispecies are collectives that have positive and negative interacting members that are bound together for a combined fitness that depends on diversity[36-38]. It is thus ironic that it is from the viruses, assumed for decades to be the most selfish of all genetic entities, we observe the characteristics of cooperative, collective behaviour. And it was the "fittest type" assumptions of Manfred Eigen[1] that generated quasispecies equations and theory which stimulated the development of this modern collective quasispecies view for over 40 years. But we are left with a conceptual contradiction. Modern quasispecies observations do not depend on the master (fittest type) and the consensus sequence. Consensus sequence may not predict the fitness of the diverse collective. In contrast to this diversity itself seems crucial.

QS-C PRODUCE HIGH RATES OF DIVERSITY: THIS IS NOT ERROR

With this clarification, it should become apparent that all RNA replicators (especially simple ones) must have high rates of diversity generation (not error). Novelty is then generated from new combinations of this diversity. Indeed, it sounds curious to use the term "error prone" for the high production rates of sequence novelties. An error is an inferior ("less better") variant of an extant sequence. Such errors should only provide rare incremental improvement and be much less (if at all) able to generate *de novo* networks. With this error concept we also apply terms such as "damage", "defect" and "incomplete" to variant information.

In contrast to this, cooperative RNA quasispecies produce and configure sequence novelties that are members of coherent populations and must generate an interacting diversity as prerequisite of innovation (variation), the driving forces of evolution. As an analogy, we might apply the limits of the "error" concept to innovative human endeavours. For example; poets produce novel poems by reconfiguring the commonly shared vocabulary. Must they also be error producers? Similarly, music composers and all artists in general produce novelty from common combinatorial rules and existing basic material tools. Do they also operate as error prone agents of fine arts? As we will see below, the qs-c concept requires interaction and diversity so it can even help us understand these processes of human innovation. But we must also apply the shared nature of "agents" that can create novelty. We will now consider how stem-loop RNAs can help us understand "agents" and their shared common use.

But in addition to stem-loop RNAs, all genetic entities that replicate via RNA will also be prone to qs-c (collective) behaviors. These behaviors will include cooperative and competitive interactions. RNA, however, is not simply providing syntax for genetic information. It is more

than code. It can also provide: (1) structure (stem-loop); (2) identity (stem-loops, 5', 3' ends); and (3) functional (ribozyme, endonuclease, ligase) activity.

And it may be dynamic (*e.g.*, pseudoknots). Because of this extended capacity relative to DNA, RNA can be considered as a more active entity, with behaviours that make it able to function as an "agent" to affect its own activity and survival[39,18]. At this point we adapt the framework from pure physics and chemistry to emergent biotic agent-based group building. In that light, DNA can be considered as a habitat for various RNA agents. It was from this perspective that we proposed that DNA should be considered as a habitat for these active RNA agents[18,32]. But this discussion of simple RNA replicators suggests that the concept of qs-c should also apply to the ideas and experiments concerning the "RNA world" hypothesis. Yet curiously, very little "RNA world" research has addressed any issues regarding quasispecies[40-42], let along the more modern qs-c idea. As many are starting to think that life originated in a cooperating situation[43], it is worth briefly considering if the qs-c concept will provide a different scenario for the origin of life.

RNA WORLD RECONSIDERED: INFECTIOUS STEM-LOOPS THAT OPERATE *VIA* QS-C

To evaluate the qs-c and infectious perspective on the RNA world hypothesis, we apply and explore the RNA-agent concept introduced above to the role of stem-loop ribozymes in the origin of life. The main objective is to incorporate the historically absent qs-c and parasitic perspective (with its inherent feature for group fitness) into the process that creates RNA societies. We will not explore early chemical evolution that might have led to the emergence of RNA molecules, but will instead assume RNA has come into existence and follow its features from this perspective.

One immediate consequence of this perspective is that we will be focused on collective features of RNA populations and will thus evaluate the chemical consequence of ribozyme qs societies, not individual replicators. This foundation immediately creates a situation in which "systems" of molecules with multiple behaviours will have the primary role in promoting the origin of life. It will also be important early on to consider how these systems maintain coherence (group identity, presented below), as this is an essential feature. Indeed, a basic and continuing theme will be that a core function of stem-loop RNAs is to provide molecular identity through all of evolution, including recent human evolution. This identity theme will persist throughout this chapter and will be frequently reintroduced.

The idea is then that individual members of stem-loop RNA societies were collectively able to invade (ligate into) each other to form a more stable and capable (ribozyme active) consortia with emergent, transformative and unpredictable abilities. These collectives would lead to the origin of various ribosome- and RNA cellular societies (still linked to its stem-loop tRNA origin). Such a scenario also introduces the basic role of cooperation in the origin of life. It does not, however, eliminate competition, preclusion or extinction which are also inherent features of qs-c behaviors. Furthermore, the identity and transmissive role for stem-loop RNAs set the early (precellular) foundation for the origin of viruses whose emergence will further drive host evolution via colonization.

The cooperative and parasitic features of qs-c will also promote the early participation of peptides in the identity and evolution of the RNPs. The maintenance of these RNPs as a coherent collective will generally be mediated by addiction modules, which underlie group identity and immunity in all living systems. Addiction modules are counterbalanced (former competing) genetic parasites which share a persistent life style in host genomes. Addiction modules are clearly the result of stable consortial interactions[4,44-46].

With this foundation, the emergence of genes, DNA, cells and individual fittest type selection can all be derived. But the emergence of DNA and cells and Darwinian evolution do not terminate the central role for transmissive RNA societies in the evolution of life. DNA becomes a habitat for these stem-loop "identity" RNAs and it is from this perspective that we can subsequently examine recent events in human evolution.

One issue should already be clear: This scenario posits that collective and cooperative behaviours were and remain essential for the emergence of living complexity. Qs-c then provides a conceptual foundation for the study of cooperating chemical biotic (in contrast to abiotic) networks in which mixtures of self-replicating RNA ribozymes can form highly cooperative and dynamic autocatalytic cycles[29,31]. Let us now put this into the perspective of virolution[11].

TO SURVIVE RNA STEM-LOOP REPLICATORS MUST FORM POPULATIONS THAT DYNAMICALLY GENERATE IDENTITY

In the origin of the RNA world, short RNA oligomers formed by chemical processes needed to become longer RNAs able to perform template based catalysis. It has been proposed that the initial chemical formation of hairpin-like RNAs (stem-loops) could provide ribozyme activity following a ligation based modular evolution that would yield ribozyme auto catalysis[47]. Indeed, below we present a series of studies that support this modular view.

But according to the parameters of qs-c evolution, for a consortium of RNA stem-loop replicators to survive, they must form a coherent population. They must share their identity and survival. The recognition of the stem-loop sequence itself by catalytic agents could provide such common identity. Alternatively, chemical markers or

initiators of catalysis could also mark the common population for priming or replication. Thus it is very interesting that the smallest ribozyme so far reported consists of just 5 nucleotides able to catalyze aminoacylation of the 3' end[48].

The addition of an amino acid to an RNA molecule has many interesting chemical implications. A ribozyme has rather limited chemical potential compared to proteins. This is mostly due to proton disassociation constant of various amino acid moieties which are not close to pH neutrality. Thus amino acids are much more capable as chemical catalyst for this reason. Without the participation of amino acids, ribozymes must attain complex folds, often with some dynamic character (pseudoknots) to be effective catalyst allowing them to cleave and ligate RNA. Given this chemical advantage, we might expect that RNA evolution was greatly facilitated (but not coded) by peptides that contribute catalytically. In addition, such a modified RNA would likely also provide a chemical marker that could distinguish this RNA population. Indeed this molecular identity idea is developed below as a way to better understand the origin of tRNA and its role in initiating replication of so many RNA viruses, as well as how this chemical marker could promote the symbolic genetic code.

BASIC GROUP-BUILDING OF RIBOZYMES THROUGH SELF-LIGATION OF RNA STEM-LOOP MODULES

A good starting point for the accumulation of complexity seems to be hairpin ribozymes whose activity can be controlled by external effectors[49]. Structural variation in these ribozymes allows progeny RNA to have different functions from their parental RNAs. The objective is to replicate RNA with RNA which hairpin ribozymes can perform as a sequence of ligation reactions that produce a longer ribozyme[50]. Along these lines, two short hairpin RNAs can catalyze their own ligation to form larger RNA constructs[30]. Thus we see interactions that promote more complex progeny. However, for a fully active ribozyme, complex RNA folding is needed. And such folding is cooperative[51].

Folded ribozymes can also interact with other small molecules promoting their function as riboswitches[52,53]. This includes amino acids which could promote either catalytic control or group identity marking. And the ribozyme folds can also be dynamic and context sensitive as seen in pseudoknots[54].

But ribozymes can also be invasive, including self invasive[55]. Thus stem-loop RNAs have many behaviors that would allow them to function as an identity group of agents involved in their own recognition and synthesis. Of particular interest is their ability to self-ligate[30] as this could promote the emergence of RNA societies with self-identity. We can also think of tRNA as stem-loop RNA with various functions and histories. Indeed, it appears that tRNAs evolved from two separate hairpins[56], in which each of the stem-loops interacts with a different ribosomal RNA subunit (presented below). This is a very interesting observation from an RNA society perspective. The invasive nature of intron ribozymes (endonuclease) also applies to tRNA from archaea, but here four distinct specificities are known[57]. This very much resembles an identity system in which introns are marking central cellular (self) agents (tRNAs) for group identity but should destroy similar tRNAs (viral, *etc.*) lacking the intron marking. It is thus also interesting that tRNA with various linked amino acids themselves have been proposed to have originated before the translation system as genomic 3' tags needed for RNA ribozyme replication[58-60]. This early function can also be explained as having served as a tag for group identity and could better explain the polyphyletic nature of the origin of tRNA[61].

Interestingly viroids, the smallest virus-like agents, which infect plants, have striking similarities to Hepatitis Delta Virus[62]. This virus, the smallest genome of any animal virus, uses circular genome, secondary structure folding and replication by a rolling circle mechanism that is catalyzed by host enzymes and cis-acting ribozymes. Mobile genetic elements are also typical self-splicing ribozymes. They excise their own RNA, from precursors thus support their own identity. The module like structure is evident. The viroids rely only on RNA and its structural motif, so it is really the sequence itself and its secondary structure which represents the entire infectious agent[62].

THE ROLES OF RNA STEM-LOOP VARIATIONS IN QS-C BUILDING

This inherent capacity to form stem-loops with loops, bulges, junctions that are not immediately repaired or corrected opens the possibility to build abundance of varieties, which alter compositional patterns, identities, immunities and the whole row of progeny within a qs-c.

Let us consider a single stem-loop RNA that undergoes several rounds of replication. Potential identity effects of diversity may integrate the following: (1) altered self; (2) new interaction with other RNA; (3) complementing replication; (4) interfering with replication; (5) serving as primer for new replication pattern; (6) serving as target for ligase; (7) serving as target for cleavage; (8) serving as target for integration; (9) serving as initiation point of strand opening; and (10) serving as interaction point with peptide.

A variety of combinations of the above listed outcomes multiplies identity-generating and identity-shifting effects (Figure 1).

Each replication event-necessarily being low fidelity-produces its own peculiar version of diversified progeny, *e.g.*, with a new "bulge" in the stem. This bulge then becomes available to provide a whole array of possible outcomes (including contradicting ones).

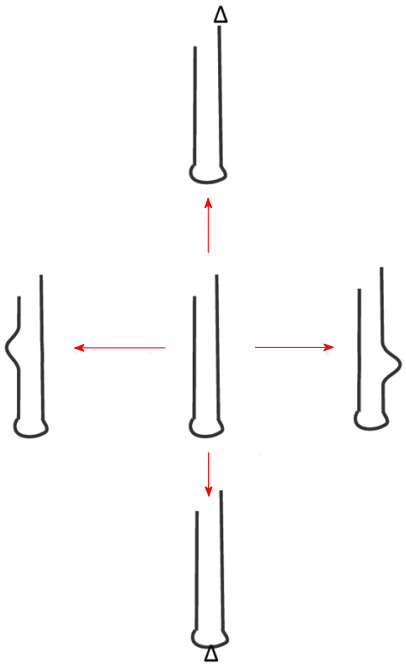

Figure 1 Self-generating RNA-stem-loop innovations: Generation of diversity (innovation) is shown. This is different from errors (accident, damage). Red arrow denotes diverse products from central template by low fidelity replication. Bulges and triangles denote changes.

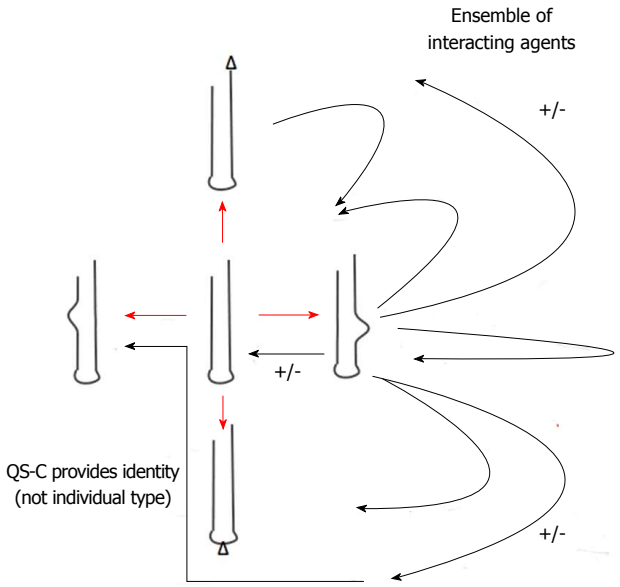

Figure 2 Crucial difference of quasispecies consortia with former quasi-species-concepts (fittest type-mutant spectra) is the basically consortial organisation of functional RNA ensembles. Shown above are the possible consortial interactions (black arrows) of just one the diversified RNA-stem-loops. These multiple activities (shown as +/-) preclude individual fitness definitions but require emergence and adaptation of group membership-identities. Defectives with similar subviral RNA-(stem-loop) groups remain relevant in both evolutionary and developmental processes. As a result of this basic evolutionary process of RNA stem-loop consortia building we can look at the emergence of *de novo* identities. QS-C: Quasispecies consortia.

It might: (1) interact with the original template to either compliment or inhibit it; (2) provide an interaction point for other RNA progeny (including itself); (3) provide a target site for cleavage or ligation; (4) act in combination with other progeny to provide more complex catalytic (ribozyme) function; and (5) alter or provide a binding site to other participants, such as peptides (RNPs).

In other words, even a singly new altered RNA now has a whole array of possible and multiple usages (positive and negative). Whole actual use will depend on the circumstances and history of the population it is in, *i.e.*, actual use depends on context.

If we add to this all the other diverse RNA progeny from these few rounds of replication-all in their own peculiar RNA region-all providing their own peculiar potential for use, we start to see the multiple potential for each individual RNA.

With such a scenario this combinations of possibilities very rapidly become too complex to follow the fate (fitness, usage) of any particular RNA. But this is the wrong, since it is an application of linear thinking. Instead, we need nonlinear thinking. Thus, if we think in sociological terms, then the RNA population (quasi species) can be considered as a "culture" that retains a common language which provides a level of group coherence (qs selection) on the basis of compatible cooperative organization. Each individual diverse RNA then becomes like a potentially new word for that language, *i.e.*, new agent in the ensemble of interacting agents.

The "culture" is then free to use a variant RNA (possibly even with multiple "meaning"), however it can also reject it. In this case, the term qs-c can have yet another meaning, c for culture (Figure 2).

These RNA uses will also vary considerable with the history of prior RNAs as well as any possible interactions with outer RNA quasi species. And the use can also vary (and be lost) with time as the culture adopts new meanings.

RNA-STEM-LOOPS AS MODULE-LIKE POOL

RNA stem-loops thus serve as multiple use and re-useable tools in RNA secondary sequence structure. Once being invented/generated such a single stem-loop may become part of another stem-loop consortium. This leads us not solely to the abundance of small and long non-coding RNAs that serve as regulatory tools in all known relevant cellular processes, but also to recombination processes that drive evolution[63-68]. As a result we then can find a changed identity of the new consortium with altered features.

FROM RNAS TO RNAGENTS: FROM ABIOTIC CHEMICAL REACTIONS TO BIOTIC GROUP BEHAVIOUR

We have seen that from single stem-loop RNA populations of diverse stem-loops that acquire multifunctional "agent" behavior (RNAgents) can be generated. The

emergence of RNAgents leads us to the emergence of life and life worlds. To understand the life-worlds of RNAgents means to understand a fascinating sphere of what was formerly not assumed to be possible: The mere sequences of molecules do not behave like physical/chemical entities in an abiotic world but behave as competent agents on genetic code syntax that cooperate and organize, constitute and innovate sequence structures[69,70]. Only groups of RNA stem-loops underly such selection. Single RNA stem-loops behave like a random assembly of nucleotides without selective forces[29,71,72]. Only if they are ligated to groups, *i.e.*, consortia, are they competent. This means they share a culture of interactional patterns and a history of defined time scales[30,73]. Both culture and history are strongly influenced by ecosphere habitats that underlie selective forces and therefore are not reproducible features of abiotic matter.

The basic module of such RNAs are their complementary composition of base pairing stems and not-base pairing loops which results of an inherent property of RNA ensembles, the fold back to complementary polyRNAs, as demonstrated by the variety of ribozymes[74]. Also the genomes of plus strand RNA viruses are able to form secondary and higher-order RNA structures[75]. Interestingly, recent experiments demonstrated that single self-replicating RNAs are not as successful as cooperative ensembles and as a result cooperative RNA consortia outcompete selfish RNAgents[31]. Also, diversity in the backbone of RNA replicators seems essential to allow strand separation and replication[76]. Thus group cooperation is key at the very origins of RNA societies (this is coherent with empirical knowledge about the emergence of natural codes/languages: they are essentially social group features not *solus ipse* results; see Witzany 1995)[16].

Additionally, such RNA stem-loop consortia as found in long noncoding RNAs take multiple roles in RNA binding, DNA-binding, conformational switch and protein binding. Their modular nature results in functions such as control regimes, scaffolding to RNPs as well as guiding in target recognition within genomes[53]. It is very clear now that non-coding RNAs build a complex layer that determines the regulation of all steps and substeps of gene control in complex organisms[18].

SINGLE RNAGENTS JOIN GROUPS AND NETWORKS: THE FEATURES

The origin of spontaneously cooperating networks of stem-loop RNA replicators can be understood from the qs-c perspective in which cooperation is the essential behavioral motif that outcompetes selfish behavioral patterns[31]. Thus we see the emergence of networks at early stages in the evolution of life. In this review the term network will be used to include some distinct features, specifically network membership. Basically, for a network to be coherent and able to act collectively, it must limit membership to promote coordination. Otherwise it is simply a collection of uncoordinated agents and there will be no selection for maintaining the network coherence or existence. If we are examining a network composed of stem-loop RNAs, it will be necessary for the individual RNAs to have some feature or behaviors that maintains membership such as RNA replication and recognition. This requires interaction. If only one type of RNA is supported (*e.g.*, high fidelity replication), there can be no complementation and complex function (*i.e.*, ribozyme) for the collective. A diversity of behavior and type will be essential. Recall however, that these RNAs act as agents in which various (multiple) behaviors will be possible even for the same sequence. This means there is diversity of interaction as well as diversity of type is inherent to the network. Thus overall interaction of an RNA agent with the collective must promote coherence and continued existence. What then are the features that promote continued existence (selection) for a network?

This does not require that only positive (*e.g.*, replication) interactions be supported. Negative interactions, including interference will also be needed. For example, highly efficient run-away replicons would overtake a qs-c and yield only one RNA type. Thus the qs would lose complementing functionality and would also consume all substrates if they were not regulated. This situation presents a problem in those habitats with limited substrates which likely is a very common state. Therefore, some level of self-regulation (negation) in the collective would promote the survival of the collective, especially if these RNAs could interact with the substrate in a regulatory, *e.g.*, riboswitch-like manner.

That efficient replicators become susceptible to parasitic replicators would provide an inherently spontaneous process of self-regulation. Yet the collective will still need to promote replication when it is favored. Accordingly, it becomes important for members of the collective to be subjected to both positive and negative self-regulation *via* RNA-RNA interactions. However, here too there must be some limits to self-regulation as the collective cannot tolerate overly active self-regulating members that will extinguish the collective. Thus we see that being a successful member of a collective requires many (and multiple) behaviors associated with it.

On top of that, as a qs-c replicates, these features will drift with time in a dynamic manner. In this context we can see that a random RNA stem-loop or a stem-loop RNA from a different qs collective would likely not be coherent with the other members of a particular qs.

A qs society is generally rather specific for its members. Group selection has already occurred in generating the qs-c. Indeed, as many experiments with RNA viruses infecting humans and animals have shown, a particular qs will exclude other qs of the same virus, such self exclusion can provide an origin for immune functions[44,77]. Such society membership is also time dependent in that the serial passage of the same viral qs will usually result in subsequent qs that preclude prior individual members. This behavior has often been called a "Red Queen" behavior, but such a classical neo-Darwinian view does not incorporate or acknowledge the issue of group membership (qs-c coherence).

GROUP MEMBERSHIP IS THE PREREQUISITE FOR SELF/NON-SELF DIFFERENTIATION COMPETENCE

An important consequence out of this perspective is that the membership view allows us to understand the maintenance of minority types in the collective since these members can provide a needed but complementing catalytic control. Thus a qs society is a network that will naturally promote the emergence of membership, not the destruction of minority types. The important side effect is, that defective interfering agents can also contribute to membership control.

As previously proposed[45,46], group membership can also be promoted by the combined action of toxic agents linked to antitoxic agents. A common version of a toxic agent is an endonuclease that will cleave sequences that are recognized as foreign. The antitoxin in this case prevents the action of the endonuclease (*e.g.*, *via* a bound protein or methylated base, dsRNA with another molecule, altered RNA fold, *etc.*). In this light, the endonuclease and ligation activities of stem-loop ribozymes are particularly interesting. A stem-loop ligase could provide a mechanism to recognize non-member stem-loop RNAs and destroy them by ligation.

Recall, however, that serial ligation can also be used to copy a stem-loop RNA. But such a situation has several very interesting implications. One of the problems with a society of stem-loop RNAs is that to attain their combined function, they need precise physical molecular placement relative to one another. This would normally require a high concentration dependence to counteract diffusion. By ligation, however, we could build a society of stem-loop RNAs that have covalently placed the various stem-loops in the correct functional (or dynamic/regulatory) context and have lost their concentration dependence. It seems likely that such a process would involve invasive self-colonizing stem-loop RNAs that results in one molecular entity with a common identity function. This would generate one entity that evolved from the ligation of a mixed set of stem-loop agents that now have a highly enhanced (collective) functional capacity. This collective would also have a highly enhanced capacity for persistence as it need not continually replicate individual stem-loop RNA agents to maintain its membership. The collective, however, would still need to oppose non-member or other parasite participation. Additionally, a collective might attain a conditional (regulated) replication capacity if it incorporates stem-loop RNA riboswitches. It is by such a process that we can now consider the origin of the ribosome.

BEFORE AND AFTER RIBOSOME EMERGENCE

A big problem with thinking that viruses are essential agents for the emergence of life, however, is the ribosome[4,9,78-81]. The ribosome really defines the cell from virus and seemed to preclude virus from early evolution of the cell[80]. It is now clear also that the ribosome acts as ribozyme[82]. Yet the ribosome itself is an ensemble of two rather complex stem-loop societies of riboagents[83], an ensemble that became "set" with the invention of DNA[84]. Therefore it makes sense now to re-evaluate the RNA virus first hypothesis[4] from the before ribosomal world (BR) and the after ribosomal world (AR). Of course, common themes of consortia, symbolic code, quasispecies, addiction modules, group identity, membership agents would provide the themes that could link the great BR-AR divide. Briones *et al*[47] developed an interesting four step model on this in scenarios of evolution on both mineral surfaces and inside vesicles, such as: (1) abiotic polymerization of RNA oligomers; (2) folding of the RNA oligomers and ubiquity of hairpin structures; (3) ligation based modular evolution of RNA and finally; and (4) template-dependent RNA polymerase. This unites the history and culture-dependent derived stem-loops integrated in the two ribosomal subunits[85].

MEMBERSHIP IS CRUCIAL FOR LIVING NETWORKS (SYSTEMS) TO EMERGE

In examining the literature relevant to qs, the RNA world and RNA network formation, we can indeed find some experimental evidence that supports qs and the spontaneous emergence of RNA networks. But almost completely lacking from such experiments is any evaluation of the membership issue. For example, quasispecies-like behavior has been observed with *in vitro* RNA replicator studies[86]. Non-enzymatic template (peptide) directed autocatalytic systems can show network behavior[87] and communities. RNA ribozyme replicator sets can also show lateral evolution[88]. Also rule-based computing simulations have been applied to similar systems in an effort to understand the emergence of parasites and antiparasites[6]. Along these lines, the hypercycle kinetic model was proposed to be a system of cross catalyzing RNA replicators which depend on cooperation for growth[3], but this is not a collective autocatalytic system as proposed above[89]. Hypercycles as proposed are not able to tolerate parasites, let alone depend on them for development. Yet the biggest problem of all such studies is that there is no assumption regarding the basic importance of network or group membership.

Without this network membership concept and its attending dynamic strategies, authentic collective action does not emerge, systems do not develop. The dynamic nature of network membership and collective action pose many unsolved problems for existing qs theory. For example, how is the multi-potential of an individual RNA to be evaluated within the qs-c if we cannot specify all the other interactions and how they change with time? We cannot apply our current ideas of fitness to this individual RNA as the historical and population context

is key. Network membership needs to be prominently considered if we are to understand the origin of the ribosome and the genetic code.

Replicator identity marking *via* 3' aminoacylated of stem-loop RNAs appears most able to explain the origin of a tRNA mediated genetic code. For in contrast to Darwinian evolution, network members will generally have multiple ancestral histories. These members will mostly originate from separate parasitic lineages that were able to penetrate defenses and join the network sometimes in mixtures. They don't need to descend from one individual or even be from the same type of agent (virus, transposon, intron, intene, *etc.*).

From this perspective we can understand why the two halves of tRNA have distinct evolutionary histories, yet tRNA is a core agent for evolution of life. Thus neither the amino acid based (peptide) ancestors nor the RNA based ancestors need a common origin to participate in a symbiogenic network. Our qs-c concept supports such a network process and-additionally-network membership provides the basis for examining noncoding RNA based regulation needed for multicellular complexity[90].

VIRAL CORE COMPETENCIES: INNOVATION, INTEGRATION, REGULATION, EXAPTATION

Because infection derived domestications, such as the whole variety of retroposon derived non-coding RNA, are now known to be transcribed and to control the regulation of genes[91], these parasitic agents shape genome architecture and function-arrangements. We now realize life on planet Earth has always resided within a virosphere and that the evolution of species depended on the virosphere features of innovation and transfer[4,39]. Humans share similar gene number as C.elegans. And humans and mice share 98.5% coding DNA. Regulatory complexity thus seems to characterize the evolution of more complex eukaryotes and stems form parasitic elements. The remaining 1.5% protein coding information is differently regulated *via* these species specific non-coding elements in increasingly complex ways. These elements act together through a variety of combinations, situations and mechanisms to re-shape genome/gene functions through epigenetic imprinting and re-printing[23]. These regulatory RNAs mostly retain the stem-loop structure. Also interesting are reports that ingestion of small RNA gene regulators seems to be usual route of RNA transfer[92].

In this perspective we have applied qs-c concept to explain complex regulatory network formation in the origin of life and the cellular protein world of higher organisms. The real species that determine all these evolutionary patterns are viruses and virus-like (infectious) RNA qs-c[32,93,94].

Viral core competencies necessitates that they be competent in host genetic and epigenetic code. These competencies provide innovation, integration, regulation, exaptation of qs consortia to form networks in the host.

A very intriguing example of this is found in placentation of mammals[10,44]. From day 1-6 post fertilization, all vertebrate embryos are similar and divide to the morula stage. But on day 6 this morula hatches and becomes a trophoblast. From here on, embryo development differs significantly. The outer cell layer is the first committed tissue of the embryo and will become the placenta. Viruses have a real affinity (tissue specificity) for this layer. This is exactly where very high endogenous retrovirus (ERV) activity is found[95-97]. This tissue has been repurposed to invade the uterine wall, suppress mother's (host) immune response, promote blood feeding (exchange), and alter mother's behaviour and physiology and brain. About 1500 placental genes are thought to have been modified by altered (ERV mediated) network re-regulation[98]. In our own view, it was likely that a collection of ERVs were involved. Other viral agents associated with reproductive biology that we don't yet understand, were also likely to have been involved.

Consider the example of human immunodeficiency virus 1 (HIV-1) as a virus that requires qs diversity and dynamics to solve very complex problems and dynamic situations. If we think of HIV-1 as a fittest type agent with only about 10000 bp of clonal RNA, such a "pure" virus could not defeat the complex human adaptive and innate immune systems. An HIV-1 limited to the "master copy" fittest type (consensus sequence from a successful human infection) would be unable to generate a qs-c, and would rapidly be eliminated by our immune system and pose no problem for vaccine development. Here, qs-c is providing fitness. It is this power of the consortia to defeat complex systems that when applied to stem-loop RNAgents can also provide the power for the generation of *de novo* diversity and cooperation needed to originate life.

CONCLUSION

In our expanding perspective from physics and chemistry to sociology, from elements/chemicals to emergence of agents, *i.e.*, from RNAs to RNAgents, we found several indices that lead us to a new concept of RNA quasispecies. In contrast to former opinions and concepts that proposed single fittest type and its mutant spectra as the mechanisms of variation that drives evolution, qs-c depend on diversity, multiuse (counter) active agents and consortia membership. Thus, when we think of a commonly shared genetic code that is used and represented by consortia of RNA stem-loops, it is providing not only information storage but active group membership-identity. Therefore we term this membership-identity qs-c. In this perspective qs-RNA and virus evolution are inherently cooperative and modular. The essential players are not fittest types, but consortia of RNAgents that need diversity.

The basic rule here is that of innovation and group selection. The modular character of consortia building with its inherent self-ligation capability of basic stem-

loop tools is based additionally on the participation and integration of defectives/mutants (errors/junk) as energy optimizing resource for module-like re-usage. In such dynamic RNA stem-loop populations several basic behavioral motifs are combined that are absent in pre-biotic chemistry; such as complementation, cooperation, competition (selection), preclusion and lethal defections. This broad range of behaviors require a diversity that also integrates the remaining "memory" (information-storing) parts of essential minorities. Under changing contexts minorities may have previously been or may yet become majorities. Thus memory of past experience is inherent in and used by qs-c.

In no other natural language are the agents that communicate (coordinate and organize) *via* repertoire of natural signs (language) also identical with the signs (words) themselves. This is precisely what we have proposed with stem-loop RNAs. This proposition defines a new phenomenon: at the beginning of life agents and "words" (information) are identical. What has been divided since invention of DNA and LUCAs (signs from agents), was formerly unified. The qs-c sociology thus describes this unified status and its interactions in their current DNA/protein habitats. Indeed it is difficult to formulate sentences about a status that never have been formulated before: Agents that represent sequences of signs are themselves subject of sequence generation (as described above). In the current RNA world (now residing in their DNA habitats) they are still alive. The contrast to the early RNA world, in that their available habitats (DNA/protein) have expanded indefinitely.

Now we can imagine the move from RNA physics/chemistry to RNA sociology. Although they consist of atoms that bind together by laws of physics and chemistry they don't behave like abiotic ensembles but as semiotic subjects absent in abiotic world. "Words" are (stem-loop) agents and "sentences" are consortia of such agents. For example, some "sentences" result in tRNAs and the ribosomal subunits which assemble to ribosomes. Some result in RNA viruses, some in defectives RNAs that serve as effective non-coding RNAs in a regulation processes of host genomes. The high mutation rate is now recognized as freedom (from mechanistic determinism) to generate new sequence (consortia) space. "Error" now becomes innovation-competence for new generations of both RNAagents and biological information with unpredictable competence and membership.

ACKNOWLEDGMENTS

The authors are grateful to Professor Vera Kolb and to the reviewers for their constructive suggestions.

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P- Reviewer: Takahashi H **S- Editor**: Song XX **L- Editor**: A **E- Editor**: Wu HL

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.4331/wjbc.v4.i4.91

World J Biol Chem 2013 November 26; 4(4): 91-101
ISSN 1949-8454 (online)

REVIEW

Cathepsins mediate tumor metastasis

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Supported by National Natural Science Foundation of China, No. 81071718, No. 81000881, No. 81372282 and No. 30973400; Fundamental Research Funds for the Central Universities, No. 21611612; Guangdong Natural Science Foundation, No. S2013010013360 and the Foundation of State Key Laboratory of Oncology in South China, No. HN2011-04
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Received: August 12, 2013 Revised: September 6, 2013
Accepted: September 14, 2013
Published online: November 26, 2013

Abstract

Cathepsins are highly expressed in various human cancers, associated with tumor metastasis. It is superfamily, concluding A, B, C, D, E, F, G, H, L, K, O, S, V, and W family members. As a group of lysosomal proteinases or endopeptidases, each member has a different function, playing different roles in distinct tumorigenic processes such as proliferation, angiogenesis, metastasis, and invasion. Cathepsins belong to a diverse number of enzyme subtypes, including cysteine proteases, serine proteases and aspartic proteases. The contribution of cathepsins to invasion in human cancers is well documented, although the precise mechanisms by which cathepsins exert their effects are still not clear. In the present review, the role of cathepsin family members in cancer is discussed.

Key words: Cathepsin; Tumor; Metastasis; Mechanism

Core tip: Cathepsins play an important role in tumor metastasis, as a superfamily, each member experts different function in tumor metastatic process. In the present, we summarized the roles of cathepsin family members and analyzed their mechanism in tumor metastasis. These provide a novel insight in tumor metastasis.

Tan GJ, Peng ZK, Lu JP, Tang FQ. Cathepsins mediate tumor metastasis. *World J Biol Chem* 2013; 4(4): 91-101 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i4/91.htm
DOI: http://dx.doi.org/10.4331/wjbc.v4.i4.91

INTRODUCTION

A multitude of processes are involved in cellular invasion and migration, including loss of cell-cell and cell-matrix adhesion and degradation of extracellular matrix (ECM) components[1]. When the expression of cell-cell and cell-matrix adhesion molecules is reduced or absent, cells lose contact with their microenvironment and are predisposed to invade and migrate into surrounding tissue. Malignant cells show increased proteolytic activity, which helps them digest the ECM. This digestion is required for cancer cells to invade and migrate through the basal lamina, which is the hallmark of malignancy[2]. In the past two decades, many researchers have focused on proteases and their role in cancer in the quest for new anticancer therapies. Proteases are a large group of enzymes that catalyze the cleavage of peptide bonds in proteins. They are subdivided into five categories: metalloproteases, including matrix metalloproteases (MMPs), cysteine proteases, serine proteases, aspartic proteases and threonine proteases[3].

Cathepsins are a class of globular proteases that were initially described as intracellular peptide hydrolases, although several cathepsins also have extracellular functions. The cathepsin family includes cathepsin A, B, C, D, E, F, G, H, L, K, O, S, V and W. Cathepsin B, C, F, H, L, K, O, S, V, W and X are cysteine proteases of the papain

Table 1 Characters of human cathepsin familymembers

Cathepsin	Subtype	Endo/exopeptidase	Expressed tissue	Function
A	Serine	Exopeptidase	Platelets, primary human antigen presenting cell, testis and epididymis	Autophagy, elastic fiber formation, platelet activation
B	Cysteine	Exo and endo	Widely distributed in macrophages, hepatocytes, renal tubules, all endocrine organs	Protein catabolism, processing of antigens hormone activation and bone turnover
C	Cysteine	Exo	Broadly distributed in tissues	Hydrolyze dipeptide esters,amides, anilides and beta-naphtylamides
D	Aspartic	Endo	Eccrine sweat, extracellular matrix and synovial fluid of cartilage	Protein degradation in an acidic milieu of lysosomes
E	Aspartic	Endo	Immune system	Antigen presentation
F	Cysteine	Endo	Antigen presenting cells	Antigen presenting
G	Serine	Endo	Polymorphonuclear leukocyte	Immune complex mediated inflammation production of angiotensin Ⅱ, degradation of extracellular matrix
H	Cysteine	Endo	Ubiquitous in cells and tissues	Endopeptidase activity
L	Cysteine	Endo	Ubiquitously expressed	Keratinocyte differentiation, protein turnover, antigen presentation
K	Cysteine	Endo	Bone, ovary, heart, placenta, lung, skeletal muscle, colon and small intestine	Bone remodeling
O	Cysteine	Endo	Broadly distributed in tissues	Protein degradation and turnover
S	Cysteine	Endo	Spleen and professional antigen-presenting cells	Ii chain proteolysis
V	Cysteine	Endo	Thymus and testis, corneal epithelium	Production of enkephalin and neuropeptide Y
W	Cysteine	Endo	T-lymphocytes	Cell-mediated cytotoxicity
X	Cysteine	Endo	Immune system	Phagocytosis, regulation of immune responses
Z	Cysteine	Exo	Widely expressed in human tissues	Protein degradation

family, and represent the largest and best-known class of cathepsin[4]. Cathepsin A and G are serine carboxy peptidases, and cathepsin D and E are aspartic proteases. Cathepsins are synthesized as inactive proenzymes and processed to become mature and active enzymes[5]. Some cathepsins are ubiquitously expressed, such as cathepsin B, L, H, and C, whereas the newly found cathepsins K, W, and X are expressed by specific cells and tissues. Historically, cathepsins were described as a group of intracellular hydrolases that participate in general protein turnover in the lysosome. However, in the last 20 years, using gene knockout models, a number of discrete functions have been identified for the cathepsin family. Specifically, cathepsin S is important for major histocompatibility complex (MHC)-Ⅱ-mediated antigen presentation[6], cathepsin L is implicated in keratinocyte differentiation[7], heart functions and reproduction[8], cathepsin K is a major factor in bone remodeling[9], and cathepsin C activates granzymes and mast cell proteases. Mutations in the *cathepsin K* or *C* genes result in the hereditary disorders pycnodysostosis and Papillon-Lefevre syndrome, respectively[10-12]. Cathepsins can be expressed at the cell surface and secreted into the extracellular space, where they can degrade components of the ECM[13]. Cathepsins are proteolytically active when attached to other cell surface proteins[14]. This extracellular activity allows cancer cells to invade surrounding tissues, blood, and lymph vessels and metastasize to distant sites. All cathepsins are synthesized as inactive precursors. The endopeptidases are activated by autolysis at acidic pH in the lysosomes and the exopeptidases are activated by endopeptidases[15]. In the present review, the roles of each member of the cathepsin family in tumor metastasis are discussed.

CATHEPSIN FAMILY AND THEIR FUNCTION

The cathepsin family includes cathepsin A, B, C, D, E, F, G, H, L, K, O, S, V and W, and their characters are showed Table 1. Cathepsin B, C, F, H, L, K, O, S, V, W, and X are cysteine proteases of the papain family, and represent the largest and best-known class of cathepsins. Cathepsin B is a lysosomal cysteine protease of the papain family of enzymes that functions as an endopeptidase and an exopeptidase[16]. The human *cathepsin B* gene is located at 8p22-p23.1[17], and the protein is widely distributed in macrophages, hepatocytes, renal tubules, gastrointestinal epithelium and fibroblasts, stratified squamous epithelium, transitional epithelium, salivary glands, pancreas, central and peripheral neuronal cell bodies, trophoblasts, and all endocrine organs[18]. It functions in intracellular protein catabolism, and in certain situations may also be involved in other physiological processes, such as processing of antigens in the immune response, hormone activation and bone turnover[16]. Cathepsin C is a papain-like cysteine protease with dipeptidyl aminopeptidase activity that is thought to activate various granule-associated serine proteases[19]. It can hydrolyze dipeptide esters, amides, anilides, and beta-naphtylamides[20]. Cathepsin C also shows transpeptidase activity[21]. Cathepsin C is involved in normal neuronal function in certain brain regions, and also participates in inflammatory processes accompanying pathogenesis in the central nervous system (CNS)[22]. The *cathepsin F* gene localizes to the long arm of chromosome 11 at 11q13. This position is the same for the *cathepsin W* gene, thereby indicating that these genes are clustered in the human genome[23]. Cathepsin

F, in a subset of antigen presenting cells (APCs), can efficiently degrade the MHC class Ⅱ-associated invariant (Ii) chain[24]. Cathepsin H, which is a cysteine protease, is ubiquitous in cells and tissues, and mainly functions as an aminopeptidase that exhibits limited endopeptidase activity. Cathepsin H is synthesized as a 41-kDa preproenzyme that is proteolytically activated through a multistep process initially resulting in a 30 kDa intermediate form and finally a single chain mature form of 28 kDa. This form can be further processed to a 22 kDa heavy chain and a 5-6 kDa light chain[25]. The human *cathepsin K* gene is encoded by approximately 12.1 kb of genomic DNA and is mapped to chromosome 1q21[26]. *Cathepsin K* mRNA has been detected in a variety of tissues including bone, ovary, heart, placenta, lung, skeletal muscle, colon and small intestine. High concentrations of cathepsin K have been found in osteoclasts and osteoclast-like cells (giant multinucleated cells)[27]. Cathepsin K is primarily responsible for the degradation of bone matrix by osteoclasts and plays a key role in osteoporosis[28]. Cathepsin L is produced as preprocathepsin L, transported *via* the Golgi apparatus as procathepsin L in secretory vesicles, and then stored as mature cathepsin L in lysosomes[29]. Intracellular protein turnover by cathepsin L is involved in several important processes, and regulation of the cell cycle may be affected by cathepsin L as it is able to degrade nuclear transcription factors[30]. In addition, cathepsin L plays a role in the immune system by degrading the Ii chain in MHC class Ⅱ processing, which is a critical step in antigen presentation. The expression of cathepsin L in the thymus was shown to be essential for the development of natural killer cells[31], cathepsin L plays a part in recycling processes during axon outgrowth and synapse formation in the developing postnatal central nervous system. When present in the acidic lysosomal compartment, cathepsin L is proteolytically active. Cathepsin O is a cysteine proteinase from the papain superfamily that is composed of 8 coding exons and 7 introns and spans more than 30 kb. The number and distribution of exons and introns differ from those reported for other human cysteine proteinases, thereby indicating that these genes are not closely related. *Cathepsin O* maps to the chromosome location 4q31-q32, which is a unique site for cysteine proteinases mapped to date[32]. Cathepsin O is expressed in all examined tissues, which is consistent with a putative role of this protein as a proteolytic enzyme involved in normal cellular protein degradation and turnover[33]. Cathepsin S, which is a cysteine protease originally cloned from human alveolar macrophages, is highly expressed in the spleen and professional antigen-presenting cells, including B lymphocytes, macrophages, and other class Ⅱ-positive cells[34,35]. Cathepsin S is essential in B cells for effective Ii chain proteolysis necessary to render class Ⅱ molecules competent to bind peptides[36] *Cathepsin V* was mapped to the chromosomal region 9q22.2, which is a site adjacent to the *cathepsin L* locus[37]. Cathepsin V shares 80% protein sequence identity with cathepsin L; however, in contrast to the ubiquitously expressed cathepsin L, its expression is restricted to the thymus and testis[38], and its uniquely high expression in corneal epithelium[39]. Human cathepsin V is involved in the production of enkephalin and neuropeptide Y, which are required for neurotransmission in health and neurological diseases[40]. The *cathepsin W* gene maps to 11q13.1 and contains 10 exons and 9 introns[41]. Cathepsin W is predominantly expressed in T-lymphocytes, specifically natural killer cells and $CD8^+$ cells, and may therefore, play a role in cell-mediated cytotoxicity[42,43]. Cathepsin X expression is restricted to various cells of the immune system, such as monocytes, macrophages and dendritic cells[44]. It is involved in phagocytosis and the regulation of immune responses, such as signal transduction, growth, maturation, adhesion, cell-cell communication, proliferation and migration of immune cells[45]. By proteolytic cleavage of C-terminal amino acids, cathepsin X regulates β2 integrin functions, impairs neurotrophic activity of gamma enolase, and the role of chemokine CXCL-12 in adhesion of hematopoietic stem and progenitor cells to osteoblasts[46].

Cathepsin A and G are serine carboxy peptidases. Cathepsin A is a serine carboxypeptidase that forms a complex with beta-galactosidase and neuraminidase. The enzyme is synthesized as a 54-kDa precursor/zymogen and processed into a catalytically active two-chain form composed of 32-kDa and 20-kDa peptides[47]. Cathepsin A is expressed in various tissues and cells such as primary human APC[48], platelets[49], testis and epididymis[50]. Cathepsin A is implicated in autophagy, which occurs after the digestion of lysosome-associated membrane protein type 2a (lamp2a)[51], and plays a crucial role in effective elastic fiber formation[52]. Cathepsin G is an endoprotease that belongs to the S1 class of serine proteases. The *cathepsin G* gene is located on chromosome 14q11.2 and has a genomic structure similar to that of neutrophil elastase. Specifically, the gene contains 5 exons and 4 introns, and spans 2.7 kb of genomic DNA. Exon 2 encodes the active site histidine, exon 3 the aspartic acid, and exon 5 the serine, which together form the catalytic triad of cathepsin G[53]. Cathepsin G, like other cathepsins, is differentially expressed within various APC types. Cathepsin G functions in primary human monocytes, B cells, mDC1, mDC2, pDC and murine microglia. In addition, purified cathepsin G can be internalized into endocytic compartments in non-expressing cells to expand their protease repertoire[54].

Cathepsin D and E are aspartic proteases. The human *cathepsin D* gene is located on the short arm of chromosome 11 in region p15 in the vicinity of the *H-ras* oncogene[55]. Three molecular forms of the proteolytic enzyme cathepsin D are found in the cell: the precursor (procathepsin D), the intermediate single-chain and the mature double-chain. Procathepsin D, which is found in the Golgi complex, is enzymatically inactive, while the intermediate and mature forms are enzymatically active and are found in the endosomes and lysosomes, respectively. The latter are involved in autophagy and apoptosis pathways, thus playing a crucial role in the control of cell and tissue homeostasis[56]. Cathepsin E is an intracellular aspartic protease of the pepsin superfamily. It is highly

Table 2 Cathepsin family members expressed in cancers

Cathepsin	Elevated in cancer	Location of cancer
A	Yes	Malignant melanoma
B	Yes	Breast carcinomas, melanoma, gastric cancer, lung cancer, colon cancer, ovarian cancer, cervical cancer pancreatic carcinomas, glioblastoma thyroid carcinoma,cholangiocarcinomas, hepatocellular carcinomas, bladder caner
C	Unclear	
D	Yes	Thyroid carcinomas, squamous cell carcinoma, renal cell, carcinoma, glioma brain tumors, laryngeal carcinoma, breast cancer, lung cancer, ovarian carcinoma
E	Yes	Pancreatic ductal adenocarcinoma, gastric cancer
F	Yes	cervical carcinoma
G	Yes	Breast cancer
H	Yes	Breast carcinoma, colorectal cancer, melanoma, head and neck carcinoma, glioma, prostate cancer
L	Yes	Breast cancer, lung cancer, gastric cancer, colon cancer, head and neck carcinomas, melanomas, gliomas, ovarian cancer, pancreatic cancer
K	Yes	Gastric cancer, squamous cell carcinoma, basal cell carcinoma, breast tumor, lung cancer, melanomas, prostate tumors, renal tumor
O	Unclear	
S	Yes	Astrocytoma, gastric cancer, hepatocellular carcinomas, glioblastomas, melanoma, gastric cancer, pancreatic islet cell cancer
V	Unclear	
W	Unclear	
X	Yes	Prostate cancer, gastric cancer, malignant melanomas, prostate cancer, lung tumors, breast cancer, colonrectal cancer
Z	Yes	Melanomas, gastric cancer, hepatocellular carcinomas, pancreatic carcinomas

homologous to the analogous aspartic protease cathepsin D. Early reports implicated the presence of an aspartic protease distinct from cathepsin D in vertebrate cells[57]. Cathepsin E is mainly present in cells of the immune system, including APC such as lymphocytes, microglia, DC, Langerhans cells, interdigitating reticulum cells and human M cells. Although cthepsin E is not present in resting B-lymphocytes, it is upregulated late in human B cell activation at both the mRNA and protein level. It has also been detected in gastric epithelial cells and osteoclasts[58].

The *cathepsin Z* gene maps to chromosome 20q13, and the protein is widely expressed in human tissues, thereby suggesting that this enzyme could be involved in normal intracellular protein degradation that occurs in all cell types[59].

CATHEPSINS MEDIATE CANCER METASTASIS

Cathepsins highly expressed in invasive tumor

Some members of the cathepsin family are highly expressed in metastatic tumors (Table 2). Cathepsin A activity in lysates of metastatic lesions of malignant melanoma was significantly higher than in primary focus lysates. Therefore, cathepsin A may play a role in metastatic dissemination of malignant melanoma[60]. Overexpression of *cathepsin B* mRNA, increased cathepsin B staining, and elevated cathepsin B activity have been found in the invasive edges of cancers, thereby suggesting that cathepsin B plays a role in tumor invasion. Cathepsin B activity is significantly elevated in a variant of the B16 melanoma with high metastatic potential tumor cells[61]. Lung cancer patients with upregulated cathepsin B tend to have higher rates of haematogenous and intrapulmonary metastases[62]. In addition, a significant increase in the cathepsin B activity in tumor-infiltrated lymph nodes was observed compared to non-infiltrated regional lymph nodes[63]. Non-small cell lung-cancer patients with high levels of cathepsin B had more frequent metastasis compared to patients with low levels of cathepsin B. This implies that cathepsin B plays an important role in tumor cell invasion and metastasis[64]. Cathepsin B enzyme activity levels are inversely correlated with the Dukes' stages. Specifically, the tumor-specific increase in *cathepsin B* mRNA content is almost 4 times greater in earlier stage (Dukes' A and B) tumors than in later stage (Dukes' C and D) tumors. Therefore, increased *cathepsin B* gene expression is a characteristic of tumors that are in the process of invading the bowel wall or local tissues, as distinct from tumors that have already spread to more distant sites[65,66]. In cervical cancer development and progression, cathepsin B expression in the invasive carcinomas was positively correlated to tumor invasion depth and lymphatic metastasis. When the *cathepsin B* gene is silenced in cervical cancer HeLa cells by siRNA, the cathepsin B expression levels of both the mRNA and protein were significantly reduced, and importantly the cell proliferation, migration, and invasion of the HeLa cells decreased significantly[67]. Cathepsin B expression increased throughout cancer progression, and gradually increased from 3- to 6-fold in low-grade astrocytoma to high-grade glioblastoma, and increased in protein abundance and enzyme activity. Therefore, cathepsin B may play an important role in human glioma progression and invasion[68]. In addition, cathepsin B directly binds to Hepatitis B spliced protein, which promotes hepatoma cell motility and invasion[69]. *Cathepsin B* knockouts retard cell proliferation and tumor growth and significantly reduce the tumor invasion[2]. Cathepsin D expressed in breast cancer cells seems to be involved in the local recurrence and metastasis formation[70]. In laryngeal cancers, neck node metastasis was significantly higher in the cathepsin D positive group

than the cathepsin D negative group[71]. High expression of lymphatic microvessel density and overexpression of cathepsin D could promote cervical lymph node metastasis in laryngeal carcinoma. Procathepsin D secreted by cancer cells, increases proliferation, metastasis, and progression of breast cancer and lung cancer[72-74]. Intense expression of cathepsin D in high-grade carcinomas may be a marker for invasive potential and aggressive behavior[75]. Among patients with positive lymph nodes, those with cathepsin D immunopositive tumor cells were at higher risk of relapsing[76]. Stromal cathepsin K expression levels were significantly higher in invasive squamous cell carcinomas (SCC) than in other epidermal tumors. Therefore, cathepsin K may play a crucial role in SCC progression by promoting extracellular matrix degradation, thereby facilitating SCC growth and invasion into surrounding tissue and vasculature[77]. Cathepsin K also was expressed in the tumour cells of all basal cell carcinoma cases and perivascular epithelioid cell neoplasms[78,79].

Cathepsins increase motility and invasion of cancer cells

In esophageal cancer invasion into the ECM, cathepsin B induction is necessary for fibroblast-mediated invasion[80]. In breast cancer, overexpression of cathepsin D results in increased fibroblast motility and invasion[81]. A matrigel invasion assay demonstrated that a cathepsin H antibody inhibited the invasion of glioblastoma cell lines[82]. Cathepsin L may increase the ability of ovarian cancer cells to invade and metastasize *in vitro*[83]. Cathepsin H affects cell migration by influencing the activity of integrins, a process that could be regulated by talin cleavage[84]. Coronin 3 has been suggested to promote the invasion and metastasis of gastric cancer both *in vitro* and *in vivo* by regulating the expression of MMP-9 and cathepsin K[85]. Coculture of *cathepsin k*+ fibroblasts enhanced the invasion of cathepsin K breast-tumor epithelial cells and this was blocked by cathepsin K inhibitors[86]. Immunostaining revealed strong cathepsin K expression in most primary melanomas and all cutaneous melanoma metastases. Therefore, cathepsin K may play an important role in melanoma invasion and metastasis by mediating intracellular degradation of matrix proteins after phagocytosis[87]. Cathepsin K contributes to prostate tumor progression in bone[88]. *Cathepsin L* knockout retarded cell proliferation and tumor growth and significantly reduced the tumor invasion[2]. Cathepsin S may serve as a useful prognostic indicator and potential target for noninvasive therapy[89]. Cathepsin S was shown to play novel roles in cancer cell migration and invasion,such as colorectal carcinomas[90], gastric cancer[91] and hepatocellular carcinoma[92]. A gene knockout approach to determine the role of cathepsin S in pancreatic islet cell cancer showed that mutants of cathepsin S impaired tumor invasion[2]. Cathepsin X upregulation was also directly associated with higher invasiveness *in vitro*[93]. Recent reports demonstrate that adhesion, migration, and invasiveness of tumor cells are dependent on the inactivation of the tumor suppressive function of profilin 1 by cathepsin X[94].

Cathepsins mediate dissemination of cancer cell

Lysates of primary malignant melanoma lesions exhibited significantly higher cathepsin A activity than dysplastic and normal pigmented nevi, and cathepsin A activity in lysates of metastatic lesions of malignant melanoma was significantly higher than in primary focus lysates. Therefore, cathepsin A may play a role in malignant transformation and metastatic dissemination of malignant melanoma[60]. In addition, cathepsin B may play a role in the dissemination of squamous carcinoma cells[95]. The reduced migration and invasion of tumor cells with reduced cathepsin B levels *in vitro* provides circumstantial evidence for a contribution of cathepsin B in tumor cell dissemination from primary tumors[96]. Cathepsin B and D are involved in AGR2-mediated dissemination of pancreatic cancer cells[97]. Cathepsin D is proposed to facilitate early phases of tumor progression such as cell proliferation and local dissemination[98]. Cathepsin L and other lysosomal proteins may play a role in the dissemination of tumor cells via the lymphatic system[99].

Cathepsins mediate degradation of ECM and collagen

Cathepsin B can directly facilitate tumor progression *via* degradation of components of the basement membrane and ECM[100] Inhibition of cathepsin B activity attenuates ECM degradation and inflammatory breast cancer invasion[101]. In addition, RNA interference-mediated knockdown of cathespin B in tumor cells reduced collagen I degradation *in vitro* and bone metastasis *in vivo*. Similarly, intraperitoneal administration of the highly selective cathepsin B inhibitor CA-074 reduced metastasis in tumor-bearing animals[102]. Thyroid carcinoma with extra-capsular invasions and metastasis had high cathepsin B activities and tended to show high type I and Ⅳ collagen degrading abilities[103]. Cathepsin B localizes to discrete cytoplasmic granules in non-invasive tumors, although it exists in a more diffuse cytoplasmic pattern in invasive tumors[104,105]. Subcellular fractionation by immunoblot and enzymatic analysis confirmed that the invasive EJ cells had active cathepsin B localized to the plasma membrane, while non-invasive RT4 cells had cathepsin B confined to lysosomes. Furthermore, immunoblot analysis revealed that invasive EJ cells contained the mature form of cathespin B, which had a molecular weight of 25 kD, while the non-invasive RT4 cells had predominantly precursor forms, which had molecular weights between 30 and 35 kDa. *In vitro* degradation assays using plasma membrane fractions isolated from invasive EJ cells and non-invasive RT4 cells demonstrated that the plasma membrane of EJ cells had the ability to degrade purified laminin, and the degradative products were similar to those obtained using purified cathepsin B[106]. Stromal cathepsin K expression levels were significantly higher in invasive SCC than in other epidermal tumors. Therefore, cathepsin K may play a crucial role in SCC progression by promoting extracellular matrix degradation, thereby facilitating SCC growth and invasion into surrounding tissue and vasculature[77]. Acidification of the local envi-

ronment caused by increased anaerobic glycolysis in cancer cells facilitates the activity of extracellular cathepsin L. In an acidic environment, cathepsin L is able to degrade components of the ECM such as collagen types I and Ⅳ, fibronectin, and laminin. Cathepsin Z can upregulate the proteins associated with ECM remodeling such as MMP2, MMP3 and MMP9, thereby implying that cathepsin Z might play an important role in hepatocellular carcinoma invasion and metastasis[107].

Cathepsins mediate angiogenesis

Angiogenesis is controlled by the balance between positive and negative angiogenic factors. It has been shown that cathepsins may influence the production and degradation of both angiogenic activators and inhibitors. There is a close relationship between the intensity of angiogenesis and overexpression of the cathepsin B protein in cancer cells in resected colon adenocarcinoma[108]. A correlation between high levels of lymphangiogenesis and cathepsin D in laryngeal carcinoma was determined, which also correlated with laryngeal carcinoma lymph node metastasis[109]. Stromal cathepsin D expression correlates with microvessel density in ovarian tumors[110]. Additionally, a significant association between cathepsin D expression in host stromal cells and vascular density has been described in breast cancer tumors[111]. Cathepsin H was identified to play an important role in the establishment and development of functional tumor vasculature and increase the metastatic potential of human hepatoma cell lines. Deletion of *cathepsin H* significantly impairs angiogenic switching of the pre-malignant hyperplastic islets and results in a reduction in the subsequent number of tumors. Furthermore, the tumor burden in *cathepsin H* null RT2 mice was significantly reduced, which correlated with defects in the blood vasculature and increased apoptosis[112]. Selective cathepsin S deficiency impaired angiogenesis and tumor cell proliferation, thereby impairing angiogenic islet formation and the growth of solid tumors, whereas the absence of its endogenous inhibitor cystatin C resulted in the opposite phenotypes[113]. The *cathepsin S* gene targeted siRNA-mediated knockdown of cathepsin S expression, lead to potent suppression of MHCC97-H cell proliferation, invasion and angiogenesis. Using the gene knockout approach to determine the role of cathepsin S in pancreatic islet cell cancer showed that mutants of cathepsin S had impaired tumor formation and angiogenesis and significantly reduced levels of tumor invasion[2]. In a syngeneic colorectal carcinoma murine model that both tumor and tumor-associated cells contribute cathepsin S to promote neovascularization and tumor growth[114].

Cathepsins induce epithelia-mesenchymal transition

Epithelial to mesenchymal phenotype transition is a common phenomenon during embryonic development, wound healing, and tumor metastasis. This transition involves cellular changes in cytoskeleton architecture and protein expression[115]. Some studies have established the importance of some members of the cathepsin family in mediating this process. A functional study found that cathepsin Z could increase colony formation in soft agar and promote cell motility. Further studies found that the metastatic effect of cathepsin Z is associated with its role in inducing epithelia-mesenchymal transition (EMT) by upregulating mesenchymal markers (fibronectin and vimentin) and downregulating epithelial markers (E-cadherin and α-catenin)[107]. Snake venom cystatin (sv-cystatin) is a member of the cystatin family of cysteine protease inhibitors. Expression of the sv-cystatin gene in MHCC97-H cells inhibits tumor cell invasion and metastasis by reducing the proteinase activity and EMT[116].

Cathepsins and cancer autophagy

Autophagy participates in tumor growth and maintenance by supplying metabolic substrate, limiting oxidative stress and maintaining cancer stem cell population[117]. Cathepsin A involves in chaperone-mediated autophagy by triggering degradation of lysosome-associated membrane protein type 2a (lamp2a)[51]. Mature cathepsin D also involved in autophagy and playing a crucial role in the control of cell and tissue homeostasis[56]. In human malignant glioblastoma M059J cells, cathepsin D functions as an anti-apoptotic mediator by inducing autophagy under cellular stress[118]. Autophagy inhibition suppresses the upregulation of XO, which is induced by cathepsin S inhibition, resulting in reduced ROS generation, DNA damage, and cell death[119]. Enhanced autophagy and reduced expression of lysosomal enzymes induced regional autophagic cell death under EBV infection in nasal natural killer/T-cell lymphomas[120]. The relationship of autophagy and cathepsin in tumor is still unclear, need to further investigate.

Cancer therapy targeting cathepsins

Cathepsin S may serve as a useful prognostic indicator and potential target for noninvasive therapy[89]. A selected cathepsin S antibody, Fsn0503, significantly blocked the invasion of a range of tumor cell lines, most significantly HCT116 colorectal carcinoma cells, through the inhibition of extracellular cathepsin S-mediated proteolysis[90]. Silencing cathepsin S expression suppressed the migration and invasion of gastric cancer cells *in vitro*. Subsequent secretomics revealed that cathepsin S silencing resulted in changes in the expression levels of 197 proteins, one-third of which are implicated in cellular movement[92]. Notably, this will enable the development of individualized treatments for cancer patients according to their cancer type and its progression. In fact, cathepsin inhibitors are already being tested in clinical trials and hold promise for combined cancer therapies[10,55].

CONCLUSION

The contribution of cathepsins in the invasion process in human cancers is well documented, although the precise mechanisms by which cathepsins exert their effect are still under active investigation. Each cathepsin member exerts different functions in tumor metastasis process:

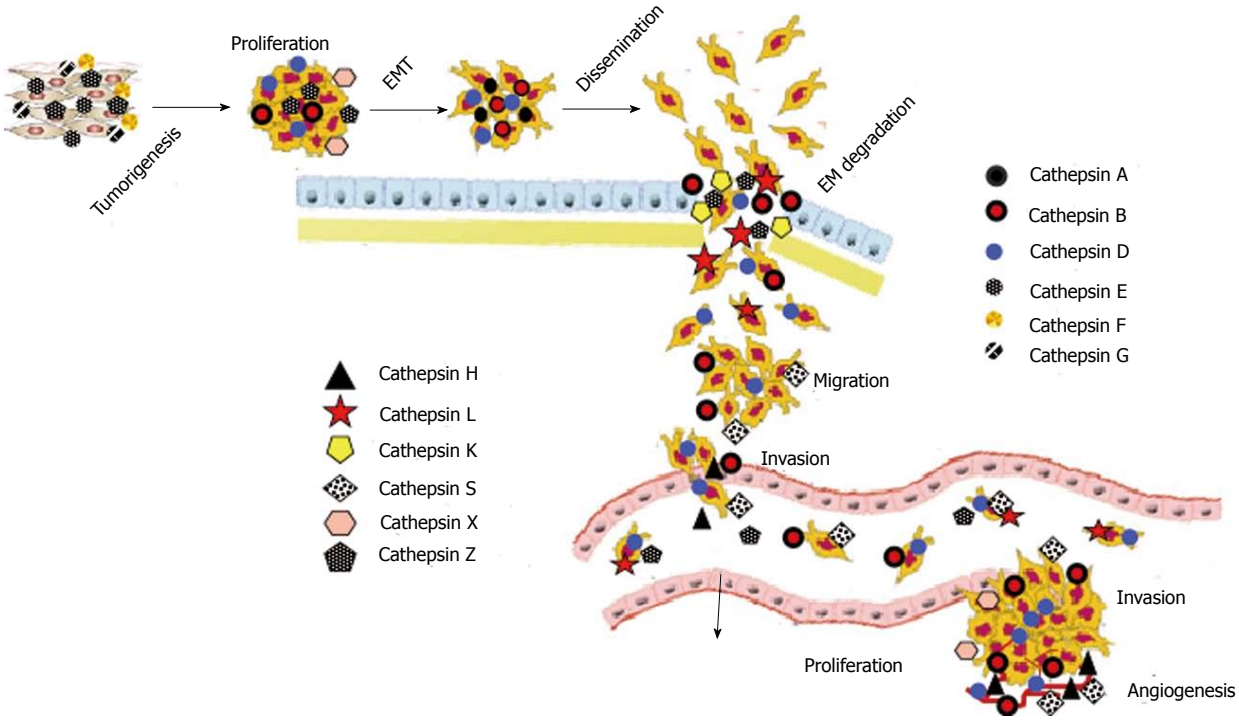

Figure 1 Role of cathepsins in tumor metastasis process. Cathepsin E, F, G, Z promote cell tumorigenesis; tumor cells are induced proliferation by cathepsin B, D, E, X; cathepsin X mediates tumor cell epithelia-mesenchymal transition (EMT); cathepsin A, B, D induces tumor cells dissemination; cathepsin B, K, L, Z degradate ECM, cathepsin B, D, H, L, S, Z increase the motility and invasive of tumor cell, and tumor cells invade to surrounding tissues, blood, and lymph vessels, and metastasize to distant sites; In metastatic sites, cathepsin B, D, H mediate tumor cell proliferation, cathepsin B, D, H, S mediate angiogenesis, and metastatic tumor forms.

(1) cathepsins can activate other proteases, thereby indirectly affecting invasion by participating in proteolytic cascades; Cathepsin B has been shown to directly activate MMP-1 and MMP-3, which in turn can cleave components of the ECM such as collagen, gelatin and tenascin; thus, facilitating the migration of tumor cells through the extracellular space[121]; (2) cathepsins directly cleave components of the BM/ECM, such as laminin, fibronectin, tenascin-C, and type Ⅳ collagen, which leads to limited proteolysis of the ECM[122-126]; (3) cathepsins can inactivate cell adhesion proteins by cleaving the cell surface protein E-cadherin, which is the principal component of adherens junctions. E-cadherin cleavage abrogates its cell-cell adhesion function and promotes tumor cell invasion; (4) cathepsins mediate angiogenesis; and (5) cathepsins induce EMT (Figure 1).

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P- Reviewers: Ding WX, Guo HB, Numakawa T **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Wu HL

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.4331/wjbc.v4.i4.102

World J Biol Chem 2013 November 26; 4(4): 102-110
ISSN 1949-8454 (online)

MINIREVIEWS

Lipidomic mass spectrometry and its application in neuroscience

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Received: August 10, 2013 Revised: September 11, 2013
Accepted: October 17, 2013
Published online: November 26, 2013

Abstract

Central and peripheral nervous systems are lipid rich tissues. Lipids, in the context of lipid-protein complexes, surround neurons and provide electrical insulation for transmission of signals allowing neurons to remain embedded within a conducting environment. Lipids play a key role in vesicle formation and fusion in synapses. They provide means of rapid signaling, cell motility and migration for astrocytes and other cell types that surround and play supporting roles neurons. Unlike many other signaling molecules, lipids are capable of multiple signaling events based on the different fragments generated from a single precursor during each event. Lipidomics, until recently suffered from two major disadvantages: (1) level of expertise required an overwhelming amount of chemical detail to correctly identify a vast number of different lipids which could be close in their chemical reactivity; and (2) high amount of purified compounds needed by analytical techniques to determine their structures. Advances in mass spectrometry have enabled overcoming these two limitations. Mass spectrometry offers a great degree of simplicity in identification and quantification of lipids directly extracted from complex biological mixtures. Mass spectrometers can be regarded to as mass analyzers. There are those that separate and analyze the product ion fragments in space (spatial) and those which separate product ions in time in the same space (temporal). Databases and standardized instrument parameters have further aided the capabilities of the spatial instruments while recent advances in bioinformatics have made the identification and quantification possible using temporal instruments.

Key words: Mass spectrometry; Lipidomics; Phospholipids; Serial signaling; Neuroscience

Core tip: Mass spectrometry offers a degree of simplicity and sophistication to the biological sciences. In this review we are focusing on its application towards the analysis of lipids in neuroscience. Lipids have a variety of functions, they surround neurons, provide insulation for transmission of signals, an environment for facilitating motility and migration of astrocytes and other cell types, among many other functions. Recent advances in mass spectrometry have enabled quantification of lipids directly extracted from complex biological mixtures in the neuronal system with the help of databases, standardized instrument parameters and bioinformatics. In this review, we intend to highlight all recent efforts with an emphasis on its application to neuroscience.

Enriquez-Algeciras M, Bhattacharya SK. Lipidomic mass spectrometry and its application in neuroscience. *World J Biol Chem* 2013; 4(4): 102-110 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i4/102.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i4.102

INTRODUCTION

The central nervous system (CNS) is lipid rich. A significant part of the dry weight of the human brain, close to

50% is contributed to by lipids[1]. Processing of behavior by organisms depends on the brain, and lipids play an active biological role towards those complex functions. Lipids provide much needed electrical insulation to the neurons enabling signal transduction through them and serving as a guiding influence in membrane fission and fusion processes. Structural properties are important for membrane protrusion and fusion. Depending on the head and tail of the individual lipids, especially phospholipids, they may form either a cone or an inverse cone[1]. It is likely that membrane fission and fusion events in neurons involve critical roles of lipids. In the fusion sites between two mating cells of protozoan *Tetrahymena thermophila*, high-resolution imaging of lipid composition using time-of-flight secondary-ion mass spectrometry has revealed that small membrane regions containing the largest number of fusion pores are highly enriched in the cone-shaped form (2-aminoethylphosphonolipid) suggesting that the localized changes in lipid geometry are likely to play critical roles in the fusion process[2]. Many lipid intermediates are potent intracellular signal transduction molecules themselves. Lipids also are central to vesicle formation, fusion and fission, all processes that are central to synaptic transmission of nerve impulses[1].

Lipids in cellular systems offer different features such as permutability of constituent fragments to generate diverse species, the capability of generating multiple types of signaling, some which can be rapid and on demand; others being localized due to their membrane bound nature. The maintenance of lipid heterogeneity across the plasma and organelle membranes facilitate lipid mediated localized signaling[1,3].

Rearrangement of structural units of individual lipids within the mammalian lipidome provides the potential for generating a vast diversity of lipid species. The capability of generating a vast number of entities provides the potential for many different types of signaling to originate from lipids. Lipid rearrangements can occur confined to a space within tissue or cells, or within the same space but at different times during the life cycle of a given cell or tissue. Lipids are capable of serial signaling, that is, application of a single biochemical route for multiple signaling events[1,3]. An example is the conversion of adenosine triphosphate into cyclic adenosine monophosphate (cAMP), which is catalyzed by adenylyl cyclase. Adenylyl cyclase is activated by binding of several neurotransmitters with neuronal surface receptors. However, the hydrolysis of cAMP stops this signaling process. Such a signaling process is a "one pathway-one signal" model of transmembrane signaling[4,5].

On the other hand, an example of serial signaling in neurons by the lipids is the 1,2-Diacyl-sn-glycerols (1,2 DAG) cascade. In this cascade, phospholipases cleave membrane bound phosphatidylinositol (PI) 4,5 bisphosphate to generate 1,2-DAG and Inositol (Ins) 1,4,5 trisphosphate. While Ins 1,4,5 trisphosphate is hydrolyzed and deactivated after signaling just as it happens to cAMP, 1,2-DAG, on the other hand often serves as the starting material for a series of competing conversions generating various intermediates. 1,2-DAG is phosphorylated into Phosphatidic acid (PA) by DAG-kinases[6]. The PA can be converted into (1) Lysophosphatidic acid (LPA) by losing a fatty-acid residue catalyzed by phospholipase A2; (2) into cytidyldiphosphate-DAG; or (3) can be dephosphorylated into 1,2-DAG by a phosphatase. 1,2-DAG can be hydrolyzed by DAG-lipases to generate Monoacylglycerol (MAG)[7,8], which itself is subsequently hydrolyzed into fatty acid and glycerol by MAG lipase[9]. 1,2-DAG could also be hydrolyzed to generate 2-arachidonoylglycerol (2-AG) and arachidonic acid, which in turn acts as substrates for lipid oxygenases[10] converting them into oxidized metabolites which are termed as eicosanoids[11]. The metabolic intermediates of 1,2-DAG, for example PA, activates raf protein kinase and PI(4)monophosphate-5-kinase[12,13]; LPA and 2-AG are high-affinity agonists for LPA and cannabinoid receptors respectively. 1,2-DAG also activates various members of the protein kinase C family[14].

It is pertinent to mention that lipids are players in neuronal retrograde signaling as well. Retrograde signaling mechanisms altering the strength of incoming synaptic inputs have been shown to be modulated by endocannabinoid lipids 2-AG or its derivatives in pyramidal neurons during their depolarization. Similar signaling is initiated by the activation of postsynaptic glutamate metabotropic receptors and acetylcholine muscarinic receptors[10,15,16].

Another important aspect of lipid based signaling is their rapid nature. Lipid signaling that can be achieved in a relatively short period of time because the signaling molecules can be generated catalytically from the existing precursor at a very accelerated pace, that which cannot be performed for larger macromolecules such as proteins. Lipids also often enable localized signaling due to their heterogeneous adherence to a specific subcellular site. Several lipids that are intermediates of the 1,2-DAG pathway serve critical functions in neuronal development, synaptic plasticity and behavior[15,17]. For example, arachidonic acid modulates the activities of neuronal ion channels[18,19]; and the eicosanoids modulate G protein-coupled signaling that underlie a myriads of neuronal functions[18,20].

The two properties, (1) dynamic changes in membrane lipids that underlie several cellular property changes; and (2) ability to generate several signaling molecules from a single precursor, simultaneously leading to multiple signaling events warrant the studying of the lipidome rather than the biochemical analyses of single lipids or only a handful of lipids. Capturing lipidome changes is therefore critical to understanding the behavior of neurons and to gather a greater insight into the functioning of the CNS. Until recently the analyses of lipids posed two challenges, (1) the inability to identify lipids without a vast arsenal of chemical reagents and a deep knowledge of chemistry; and (2) the necessity of a large amount of purified lipids for structure determination. However, the advent of mass spectrometry has largely removed these two barriers. We present here an overview of mass

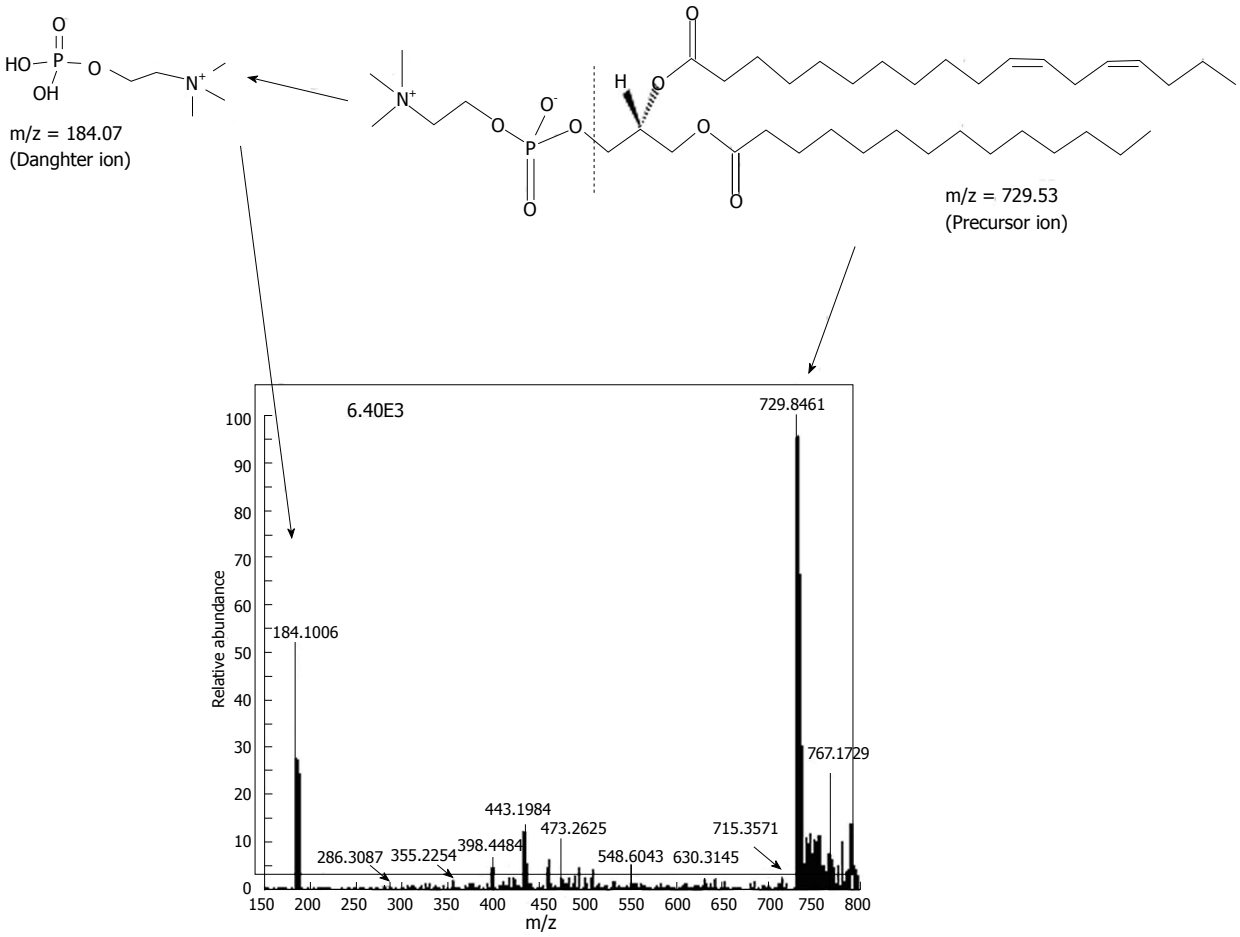

Figure 1 Electrospray ionization mass spectrometer of 1-tetradecanoyl-2-(11Z,14Z-octadecadienoyl)-sn-glycero-3-phosphocholine (m/z 729 for singly charged species). The scan was performed in the third quadrupole (Q3) of a Triple stage quadrupole Quantum Access Max instrument with collision energy of 35 V in Q2. The precursor (or parent) ion and daughter ion have been shown in the spectrum. The corresponding molecular structures of daughter ion (184.07) and precursor ion (729) have been shown above the spectrum and corresponding m/z in the spectrum has been identified by dashed arrows. The total ion intensity was 6.4E3 as indicated. The arrow head and thick dashed arrows in the precursor ion structure indicate the fragmentation point leading to the generation of the daughter ion.

spectrometric techniques enabling the identification and relative quantitation of cellular lipidome (categorized into class specific and pan lipidome analyses) and briefly discuss their utility for neuroscience related research.

CLASS SPECIFIC ANALYSES OF LIPIDS ON MASS ANALYZER WHERE FRAGMENTS ARE RESOLVED IN SPACE

Electrospray ionization mass spectrometry enables the identification and quantification of the cellular or tissue lipidomes directly from crude extracts of cell, tissue or organ samples[21-23]. The mass spectrometers that resolve fragments of entrant precursor ions of biomolecules in space domains enable identification and quantification in a class specific manner, referred to as automated shotgun lipid profiling[24]. Mass spectrometers where fragmentation and analysis occur in separated chambers (different chamber than that of the entrant precursor ions) enable precise control of collision energy and hence enable class specific scans for lipids with one class scan at a time. Ion mode and collision energy are the two key parameters which remain somewhat standardized for class specific identification of lipids among different instrumenPt platforms. The knowledge of class specific parameters has been derived from control experiments with purified lipids from many lipid chemistry groups over several decades of research in synthetic organic chemistry. The most common of space resolving instruments are the triple quadrupole mass spectrometers, Figure 1 shows a typical spectra. The instrument parameters vary on instruments manufactured by different vendors (referred to as platforms). The settings of other parameters for class specific identification need fine tuning from one platform to another and enable quantification of lipids on moderate and higher resolution triple quadruple mass spectrometers with only little optimization. The moderate resolution space domain resolving instruments (approximately 1amu resolution) enable lipid profiling from extracts of crude biological samples. The specific lipid entities of interest may then be confirmed for identities using a combination of high resolution instruments and chemical derivatization for identification of correct isomers.

Table 1 Comparison of leading mass spectrometers for lipidomics

	Resolution	Mass range (m/z)	Mass accuracy (ppm)
ToF			2-5 typical
Waters LCT premier	10000	18000	NA
Agilent LC/ToF	> 22000	50-20000	< 1
Bruker microToF	10000	3000	NA
QToF			Same as ToF
Agilent 6540	> 40000	50-10000	< 1
Waters QToF ultima	17500	32000	1
Waters QToF micro	5000	20000	1
Bruker max is 4G	> 60000	10000	< 1
LTQ velos	3000	4000	0.1 Da
LTQ-orbitrap velos	> 100000	4000	< 2
TSQ quantum access max	0.4 (FWHM)	3000	0.1 Da
TSQ quantiva	0.2 (FWHM)	1850	0.1 Da
Q-exactive	140000	6000	< 1
Orbitrap fusion tribrid	450000	4000	< 1
Synapt G2-HDMS	40000	32000 (100000[1])	< 1
Synapt G2S-HDMS	20000	32000 (100000[1])	Approximately 2

[1]When fitted with a quadrupole. m/z: Mass to charge ratio, ppm: Parts per million; LCT: Liquid chromatograph; LTQ: Linear trap quadropole; TSQ: Triple stage quadrupole; HDMS: High definition mass spectrometry; FWHM: Full width at half maximum; ToF: Time of flight; NA: Not available.

A comparison of different mass spectrometers used for lipidomics has been provided in Table 1. With the advent of knowledge of lipid chemistry, further advancement has been made to identify lipid side chains using mass spectrometry and also to incorporate a second quantification step. The parameters for these determinations have been presented in Tables 2 and 3. Three chief parameters contribute towards class specific identification of the lipids: (1) ion mode of operation (positive or negative); (2) collision energy (CE usually in volts); and (3) scan type, that is, precursor-ion (often also referred to as parent-ion) or neutral loss scan for the daughter ions that is generated due to the collision induced dissociation of the parent ion. The class specific identification and quantification is based on the scan type due to generation of daughter ion of specific mass under a given collision energy[24]. All mass spectrometers usually are equipped with software for data acquisition. The optimal parameters for a specific instrument platform are not universal but need to be tuned for another platform. A lipid species may belong to more than one class due to presence of common parts encompassing more than a class, thus enabling their analysis using more than one class setting. The identification of lipids in these instruments necessitates comparison of experimental spectra to a reference spectrum or comparison of m/z ratios to databases. These comparisons are achieved through the use of analytical softwares.

In mass spectrometric lipidomics, the quantification of lipids is ratiometric and performed with a synthetic or purified standard, often termed as internal standard usually in a two-step process[23-25]. The most abundant lipid species are quantified using direct comparison of the peak intensities to those of the added internal standard for the lipid class in the mass spectrum from the first step. This is done after correcting for the isotopic differences of ^{13}C, which are often present in low abundance in the internal standard molecules[25]. The basic premise of this quantification is that the lipid species ions linearly correlate with its concentration in the low-concentration region of the mass spectrometer (MS) spectrum[25,26]. In the second step, the ratiometrically determined concentrations of the abundant lipids using internal standard are used for additional refinement of quantification. Different lipids of the same class may undergo different fragmentation kinetics[26], thus multiple standards belonging to a single class and representing different physical properties (side chain and degrees of unsaturation) enable better quantification[27-29]. Ratiometric quantification encompasses the entire linear dynamic range of ion peak intensities offered by the spatial resolving instruments and a two-step approach enables quantification of low abundance region of lipid species from organic extracts. However, one of the limitations of class specific identification using spatial instruments is the ready identification of entities that are present in the databases. The structures of lipid entities that are absent in databases can also be deduced particularly with different collision energies or utilizing high resolution mass spectrometers with chemical derivatization.

ROLE OF DATABASES

Databases play an important role in the identification of lipids in the class based lipid identification approach. Databases are usually built on either single instruments or very closely related instrument platforms, with most parameters being normalized, except for variation in parameters that enable determination of the given lipid class (Tables 2 and 3). The parameters that could be normalized are sheath gas flow rate and spray voltage, while the collision energy and mode (negative or positive ion) are parameters which have to remain largely unaltered for class based lipid identification. The limitation of identification in this approach thus depends on the presence of a given entry in the database. To a certain extent, platform dependency is not a limiting factor. Most often, normalization of analytical parameters across instrument platforms is achievable and verifiable with known standards that enable validation of class specific identification on different instrument platforms, thus enabling class specific lipid identification.

BIOINFORMATICS APPROACHES AND SOFTWARE

There exists a few databases for lipids such as the yeast metabolomics and human metabolomics databases (www.ymdb.ca and www.hmdb.ca respectively). The most complete and easily downloadable database is lipidmaps database (www.lipidmaps.org), which enables download-

Table 2 Select performance parameters of some commercially available mass spectrometers

Parameter	TSQ quantum access max	TSQ quantiva	LTQ-orbitrap velos	Qq-ToF	Synapt G2-S HDMS	Q-exactive	Agilent 6540	Orbitrap fusion tribrid
Acquisition rate	5000 amu/s	15000 amu/s	1/s@60000 RP 3/s@15000 RP	3/s@30000 RP	30/s MS or MSe mode 2000/s IMS mode	13/s@17500 1.5/s@140000	20 MS/s 10 MS2/s	15/sec@15000
Linear dynamic range	5 orders	6 orders	5-6 orders	2-3 orders	> 5 orders In-spectrum[1]	> 5 orders	5 orders	> 5 orders
Mass accuracy	0.1 Da	0.1 Da	< 3 ppm MS < 3 ppm MSn (no lock mass)	< 5 ppm Full scan w/Lock Mass > 100 ppm MS/MS	< 1 ppm MS MSe or MS/MS	< 1 ppm	< 1 ppm MS < 2 ppm MS2	< 1 ppm < 1 ppm MSn
Resolution	0.4 Da FWHM	0.2 Da FWHM	> 100000 RP	Up to 30000 RP	Up to 40000RP	140000	> 15000 RP@ 1522	450000
Sensitivity	Sub-femto-mol	Atto-mol	Sub atto-mol	Sub femto-mol	Approximately 10 charges at the detector	Atto-mol	Femto-mol	Sub atto-mol

[1]At full resolution and maximum acquisition rate. ToF: Time of flight; LTQ: Linear trap quadropole; TSQ: Triple stage quadrupole; HDMS: High definition mass spectrometry; MS: Mass spectrometry; IMS: Ion mobility spectometry.

Table 3 Class specific scanning parameters of different lipid classes using spatial resolution triple quadrupole mass spectrometer[1]

Lipid class	Ion mode	Class-specific pre screen			Second-step quantification		
		Daughter ion mass (m/z)	Collision energy (V)	Scan type	Daughter ion mass (m/z)	Collision energy (V)	Scan type
PC	+	184	35	PIS	189, 183.1, 59	35, 35, 24	NLS
lysoPC	+	59	22	NLS	205, 59	34, 22	NLS
PE, lysoPE	-	196	50	PIS	222.2	30	NLS
PI, LysoPI	-	241.1	45	PIS	241.1	45	PIS
PS, lysoPS	-	87.1	24	NLS	87.1	24	NLS
PG, lysoPG	-	153.1	35	PIS	153.1	35	PIS
PA, lysoPA	-	153.1	35	PIS	153.1	35	PIS
CL, monolysoCL	-	Full MS at high resolution					
Triglycerides	+						
Spingomyelin	+	213.2	50	NLS	213.2	50	NLS
Ceramide	-	256.2	32	NLS			NLS
Sphingoid base	+	48	18	NLS	48	18	NLS
Psychosine	+	180	24	NLS	180	24	NLS
Acyl -carnitine	+	85.1	30	PIS	85.1	30	PIS
Acyl-CoA	-	134	30	PIS	134	30	PIS

[1]The parameters have been adopted from a review of vast literature, which has been reviewed in Yang *et al*[24]. Some lipid extraction for analyses in positive ion mode performed in presence of Sodium or Lithium ion will change the mass to charge ratio (m/z) of ions. Similarly, extraction performed for analyses in negative ion mode in presence of ammonium acetate may change the m/z of ions. MS: Mass spectrometry; PIS: Precursor ion scan; NLS: Neutral loss scan; PC: Phosphocholine; lysoPC: Lysophosphocholine; PE: Phosphoethanolamine; PI: Phosphoinositol; PS: Phosphoserine; PG: Glycerophosphoglycerols; PA: Phosphatidic acid; CL: Cardiolipin.

ing specific lipid class entries in *.csv and a few other formats that can then be locally used for searching using different softwares. A few different softwares are available. Multidimensional mass spectrometry-based shotgun lipidomics (MDMS-SL) and MZmine (recent version 2.10.0) are freely downloadable while Simlipid 3.0 is a commercially available software. Ratiometic quantification in these softwares is achieved as a two-step process. In the first step a specific class based known standard that does not naturally exist in mammalian systems is first used for quantification of the most abundant ion species within the lipid class in the previously identified entities. In the second step the standard and abundant lipids are used for quantification of low abundance species in the experimentally obtained ion spectrum.

TOTAL LIPID ANALYSES AND *DE NOVO* LIPID IDENTIFICATION AND QUANTIFICATION USING MASS ANALYZERS THAT RESOLVE FRAGMENT IONS IN TIME ON THE SAME SPACE

Many instruments harboring ion-trap type of mass analyzers enable generation of fragment ions from precursors in the same space but over different time spans and are termed time resolving instruments. Time resolving mass spectrometers enable the capture of ions with minimal loss as ions are not lost in a vast space that they would have to travel otherwise. The ability to acquire and

align multiple related high resolution spectra enables *de novo* analyses of new species that may not be present in the database. The acquisition and alignment of related MS/MS spectra reduces the false positive assignments and greatly improves the ion statistics. Time domain resolving instruments capture all precursors and their fragments in parallel and in a single scan. However, they utilize a single collision energy that fragments different lipids with different efficiencies. The analyses of the data involves relating the fragments to their precursors, a task that poses a great challenge. The analyses of such data is handled by bioinformatics. Another approach is specific chemical derivatization that selectively reacts to specific or class specific lipids eliminating them from the total spectra upon chemical derivatization. Thus, mass spectra with and without chemical derivatization enables distinguishing specific lipids from scans performed in time domain resolving instruments[30-32].

The time domain instruments produce a comprehensive dataset of MS precursor ions and the MS/MS spectra comprising all fragment ions derived from all lipid precursor ions[33]. In these instruments a survey or MS spectrum is acquired to determine m/z and abundances of precursor ions, which follows acquisition of MS/MS fragment spectra from automatically selected precursors. The acquisition of MS and MS/MS spectra is repeated. Each acquisition comprises a large number of MS and MS/MS spectra from selected precursors. The MS and MS/MS spectra share common attributes: (1) mass accuracy (ppm, Da or amu); (2) mass resolution (FWHM); and (3) occupancy of peaks. Mass accuracy and mass resolution are properties of individual mass spectrometers and applies equally to all peaks. The peak occupancy is dependent on: (1) instrument performance; and (2) intrinsic characteristics of the sample. Repetitive acquisitions do not often fully compensate for low abundant precursors, which are often affected by poor signal-to-noise ratio. The low abundant precursors are often not fragmented in all acquisitions and often occur with non-equal efficiency. The peak occupancy attribute is the frequency with which a particular peak is encountered in individual acquisitions within the full series of acquisitions[34]. Normalizing for peak occupancy is often used for enhancing coverage and reproducibility of peak detection.

BIOINFORMATIC APPROACHES AND INSTRUMENT INDEPENDENT IDENTIFICATION OF LIPIDS

As stated above, many freeware (for example, MZmine 2.10) as well as commercial software (Simlipid 3.0) exist for analyses of mass spectrometric lipid identification. A number of programs have been developed for analyses of MS and MS/MS datasets from high resolution and time domain mass spectrometers. LipidXplorer is a recently developed program that has been built from the experience of several previous programs[34]. The LipidXplorer software is based on three axioms: (1) that the software should utilize spectra acquired on any tandem mass spectrometer; (2) should identify and quantify species from any lipid class; and (3) should handle large datasets composed of highly redundant MS and MS/MS spectra, with several technical and biological replicates acquired from each analyzed sample. LipidXplorer therefore enables *de novo* or database-independent identification of lipids. Spectra interpretation rules are flexible and not encoded into the software engine. In LipidXplorer users can define new rules or modify the existing rules and apply any number of interpretation rules in parallel. LipidXplorer employs a two-step process for lipid identification employing a two-step analyses in a fully database-independent manner[34,35]. In step 1, a full pool of acquired MS and MS/MS spectra is organized into a single flat-file database termed as MasterScan. In step 2, the MasterScan is interpreted using molecular fragmentation query language (MFQL). The findings of MFQL are exported in a user-defined format. These two steps eliminate the reliance of comparison of experimental and reference spectra for lipid identification.

CHEMICAL MODIFICATION APPROACHES FOR COMPLEMENTARY CONFIRMATION.

Chemical derivatization using reagents that are relatively specific in reacting with one group versus others enables selective elimination of peaks in a given spectra and thus allow identification of those species. Derivatization is used for conversion of nonpolar or less polar lipids into polar lipids in electrospray ionization mass spectrometry[36,37]. The conversion into polar lipids necessitates imparting an inherent charge. Addition of sulfate group to cholesterol[36] or oxosteroids into their oximes[38] are examples of rendering these entities polar. Derivatization enhances detection of specific lipids or members of lipid classes on time domain resolved instruments. Derivatization also often assists localization of lipids on cell and organelle membranes or even identification of lipid bound proteins in the membranes[31,32,39].

UTILITY IN NEUROSCIENCE

Mass spectrometric lipidomic analyses is poised to identify specific lipid players at different locations of neuronal cells of various types that serve diverse specialized functions. One of the key feature of neurons is (1) insulation, a process so central to neuronal function in neurotransmission. The insulation in neurons is achieved by the association of lipids with proteins[40,41]. For example, Myelin basic protein's organization and proper functioning are optimized at a particular combination of both the amounts of and ratio between the charged lipids and Myelin basic protein[42]; (2) another important biological aspect of neurons is transport across rather long

region of the body of cells termed soma. Neuronal cells employ a wide variety of molecular motors[43] and the hindrance to axonal transport is part of the pathology in several neurological diseases. In this context, the role of specific lipids remains very important to analyze. Mass spectrometric detection and quantification of lipids associated with various transport components will provide a rare insight into the biological chemistry of neuronal transport; and (3) is signaling. In neuronal systems signaling occurs broadly in three different ways: (1) within the cells of similar types, the connected neurons are an example of this; (2) with the cells of dissimilar types, for example amacrine cells signaling to neurons[44,45]; and (3) with environmental factors serving as environmental cues (for example, trophic factors[46]) to neurons. These signaling events are important for neuronal patterning in development, their maintenance in adulthood, the health and day to day function of the organism responding to the opportunities and threats in the environment and in health and disease, when their aberrations results in progressive neurological deficits. For example, a form of retrograde signaling is initiated by a voltage-dependent influx of Ca^{2+} into hippocampal neurons that leads to the inhibition of glutamate-mediated or γ-aminobutyric acid (GABA)-mediated inputs[16,47,48]. This retrograde signaling mechanism appears to be widespread in the CNS, and is mediated by a diffusible endocannabinoid lipid[49,50]. The endocannabinoid 2-AG has been directly implicated in mGluR-induced retrograde signaling in the hippocampus, cerebellum and other regions of the brain[51,52]. 2-AG is likely also produced in dendritic spines *via* a phospholipase C/DAG lipase (DGL) pathway. The fidelity of the 2-AG mediated signaling sequence depends on the precise localization of DGL-α. The latter is the major biosynthetic enzyme for 2-AG in neurons[7,53]. Some lipid messengers use stable signaling junctions. In the brain, most lipid signals such as 2-AG travels short distances from their sites of production and engage G-protein-coupled receptors on neighboring neurons and/or glial cells. The lysophosphatidic acid[54,55], platelet-activating factor[56,57] and anandamide[15,58], a neurotrophic signal that play a role in neuropathic pain, a retrograde messenger in hippocampal long-term potentiation, and an endocannabinoid ligand have already been identified as lipid messengers for signaling. Neurosteroids, oleoylethanolamide and its derivates are some examples of lipid messengers that do not require G-protein-coupled receptors to exert their function. Neurosteroids interact with membrane GABA-gated receptor channels to enhance neuronal inhibition[59]. The oleoylethanolamide and its analogue palmitoylethanolamide regulate peroxisome proliferator-activated receptors-α in the cell cytosol and nucleus that contribute to feeding[60] and pain sensations[61].

CONCLUSION

Lipids are involved in almost all steps of critical function of nervous systems. Several lipid changes occur simultaneously that regulate the membrane structure and function which underlie the function of neurons at the cellular level. Lipids also are involved in serial signaling resulting in multiple signaling with single precursor molecules. It is important therefore to understand changes in several lipids simultaneously. Advances in mass spectrometry have made such investigations simpler by allowing to profile and quantitate lipids simultaneously from complex biological mixtures of cells, tissues or organ extracts. Both, class specific identification of known lipids based on database as well as *de novo* identification of new lipids are possible by judiciously utilization of different types of mass spectrometers.

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P- Reviewers: Bian ZX, Masocha W, Osaka H **S- Editor:** Gou SX
L- Editor: A **E- Editor:** Wu HL

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.4331/wjbc.v4.i4.111

World J Biol Chem 2013 November 26; 4(4): 111-118
ISSN 1949-8454 (online)

MINIREVIEWS

Adaptive and maladaptive expression of the mRNA regulatory protein HuR

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Received: June 28, 2013 Revised: October 1, 2013
Accepted: November 2, 2013
Published online: November 26, 2013

Abstract

The RNA-binding proteins involved in regulation of mRNA post-transcriptional processing and translation control the fates of thousands of mRNA transcripts and basic cellular processes. The best studied of these, HuR, is well characterized as a mediator of mRNA stability and translation, and more recently, as a factor in nuclear functions such as pre-mRNA splicing. Due to HuR's role in regulating thousands of mRNA transcripts, including those for other RNA-binding proteins, HuR can act as a master regulator of cell survival and proliferation. HuR itself is subject to multiple post-translational modifications including regulation of its nucleocytoplasmic distribution. However, the mechanisms that govern HuR levels in the cell have only recently begun to be defined. These mechanisms are critical to cell health, as it has become clear in recent years that aberrant expression of HuR can lead alternately to decreased cell viability or to promotion of pathological proliferation and invasiveness. HuR is expressed as alternate mRNAs that vary in their untranslated regions, leading to differences in transcript stability and translatability. Multiple transcription factors and modulators of mRNA stability that regulate HuR mRNA expression have been identified. In addition, translation of HuR is regulated by numerous microRNAs, several of which have been demonstrated to have anti-tumor properties due to their suppression of HuR expression. This review summarizes the current state of knowledge of the factors that regulate HuR expression, along with the circumstances under which these factors contribute to cancer and inflammation.

Key words: HuR; RNA binding proteins; Transcription; RNA stability; Translation; Protein stability; Cell stress; MicroRNAs; Cancer

Core tip: HuR is an RNA-binding protein that regulates post-transcriptional processing of thousands of mRNAs, including many that encode proteins that are critical to basic cellular functions. Thus, while loss of HuR can lead to cell death, pathological overexpression of HuR is associated with numerous types of cancer. However, the mechanisms that govern expression of HuR have only begun to be delineated. This review summarizes the current state of knowledge of these mechanisms and how they may contribute to cell survival and pathology.

Govindaraju S, Lee BS. Adaptive and maladaptive expression of the mRNA regulatory protein HuR. *World J Biol Chem* 2013; 4(4): 111-118 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i4/111.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i4.111

INTRODUCTION

HuR is a ubiquitously expressed RNA-binding protein (RBP) of the embryonic lethal, altered vision (ELAV) family, and is one of the best-described regulators of

mRNA fate. HuR produces broad cellular effects by binding to its target mRNAs, which number in the low thousands, and by aiding in mRNA splicing, stability, and most often translation, although a small subset of targets are translationally repressed by HuR. In recent years, transcriptome analysis has identified the mammalian mRNA targets of HuR[1,2], and has further identified HuR as a master regulator of other RNA binding proteins[3]. Because of its broad effects on so many aspects of post-transcriptional gene control, HuR may be considered as a "regulator of regulators"[3].

HuR binds its mRNA targets through sequences rich in uridine or adenosine/uridine (AREs), which are most typically present in non-coding regions of the transcript, particularly introns and the 3' untranslated region (UTR). Under normal growth conditions, HuR is present primarily in the nucleus, but can shuttle to the cytoplasm to aid in mRNA processing. Translocation to and sequestration in the cytoplasm occurs under conditions of cellular stress (*e.g.,* UV irradiation, nutrient or energy depletion, heat shock[4-7]) where it is believed to aid in coordinating mRNA turnover in a manner that protects cell survival[8]. HuR is also a potent promoter of cell proliferation and survival[9], but during lethal stresses, can aid in promoting caspase-mediated apoptosis[10]. Thus, HuR activity is critical for regulating pathways that mediate cell survival and death.

Aberrant overexpression of HuR can lead to cellular transformation, and indeed, heightened HuR levels have been observed in tumors from tissues throughout the body. Thus, tight regulation of HuR expression is key to promoting healthy cell survival while at the same time preventing pathological proliferation. Not surprisingly, regulation of HuR expression is intricately controlled at multiple levels of transcriptional, post-transcriptional, translational, and post-translational control. Here we will review the current state of knowledge of these mechanisms and discuss how HuR expression is controlled in physiologically adaptive ways such as response to cellular stress, and in situations that lead to pathological proliferation of cells and tumor formation.

PHYSIOLOGICAL EXPRESSION OF HUR AND RESPONSES TO STRESS

Regulation of HuR mRNA expression

The first studies of genetic regulation of HuR were performed in 2000 when the 5' region of the mouse *HuR* gene was isolated and mapped. Primer extension experiments using mRNA from various tissues and cell lines revealed three products, suggesting the presence of multiple alternative transcriptional start sites. A *Spe*I-*Sma*I restriction fragment containing most of exon I and a few hundred bases of upstream sequence was demonstrated to contain transcriptional activity in reporter assays[11]. However, a definitive transcriptional activator was not identified until 2008, when it was revealed that HuR expression was mediated through the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)/nuclear factor kappa B(NF-κB) pathway[12]. An NF-κB binding site in the HuR promoter was described as starting 133 bases upstream of the transcriptional start site, although a specific start sequence was not specified. Nonetheless, the activity of this binding site was clearly proven in various gastric carcinoma cell lines. Later studies from our own laboratory confirmed that PI3K/Akt/NF-κB regulation of HuR is also present in renal proximal tubule cells, and that this pathway is one arm of a positive feedback loop that results not only in transcriptional activation of HuR, but also in continued increases in Akt activity[13]. Therefore, without a "braking" mechanism for this signaling pathway, heightened levels of Akt and HuR can lead to tumorigenic conditions, as will be described below.

A second level of regulation for HuR was identified as a consequence of the use of alternate transcriptional start sites. Our work on the role of HuR in renal proximal tubule cells during metabolic stress had identified two transcriptional start sites, at approximately 150 and 20 bases upstream of the coding region[14,15]. The 5' UTR of these alternate transcripts are very different; the longer mRNA contains a G+C-rich 5' UTR with a great deal of predicted secondary structure, while the shorter mRNA contains an A+T-rich sequence with very little secondary structure. *In vitro* translation assays demonstrated the shorter mRNA to be much more readily translated than the longer form[14]. During normal growth the alternate transcriptional start sites were used at roughly equal frequencies, but following metabolic stresses to kidney cells such as thapsigargin treatment or energy depletion, expression of the shorter transcript was increased[15]. Expression of this transcript was found to be regulated by multiple Smad 1/5/8 binding sites that were present in the 5' UTR of the longer transcript. Expression from these sites was further shown to be responsive to bone morphogenetic protein 7 (BMP-7), which notably is a key regulator of renal development and recovery from ischemic stresses[16-20]. These findings suggest that metabolic stresses may prime cells to synthesize a more readily translatable form of HuR mRNA to aid in cell survival. Figure 1 depicts the transcriptional mediators and Akt activation pathway that lead to increased HuR mRNA expression.

Production of transcripts with alternate 3' UTR due to multiple polyadenylation sites is common in both rodents and humans, and the choice of polyadenylation sites may be used to achieve a specific biological outcome. In many cases, this choice can produce either a long 3' UTR that contains AREs or a shorter 3' UTR that lacks AREs[21]. In this way, it is expected that mRNAs from a single gene may be produced with lesser or greater stability. The *HuR* gene itself encodes two polyadenylation variants, a longer and more labile form containing functional AREs, and a shorter, more predominant form that lacks AREs[22]. It was subsequently demonstrated that HuR autoregulates its expression by virtue of control over the production of these variants. Briefly, HuR regulates its own expression through a negative feedback loop[23]. Nuclear HuR can bind its own pre-mRNA and

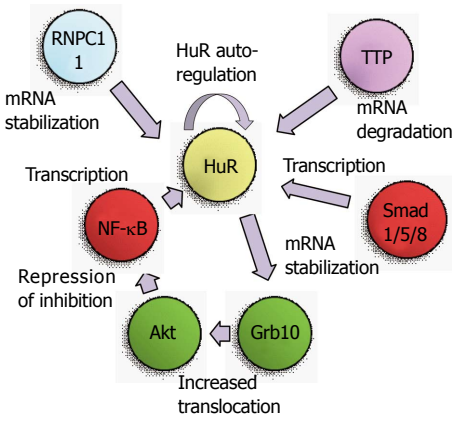

Figure 1 Translational and post-translational regulators of HuR protein levels. TTP: Tristetraprolin; Akt: Protein kinase B; NF-κB: Nuclear factor kappa B.

increase production of the longer, more labile variant, thus keeping HuR levels at constant and relatively low physiological levels. These results also suggested that under conditions in which HuR is primarily cytoplasmic, the negative feedback loop may be interrupted, thus leading to increased HuR levels and potential oncogenic transformation of cells. Under some circumstances, HuR may also serve as a positive regulator of its own expression, as a role for HuR has been proposed in facilitating export of HuR mRNA from the nucleus in senescent cells[24]. More recent studies have implicated the RNA-binding protein RNPC1 as a mediator of HuR mRNA stability. RNPC1, like HuR, can bind to AREs and regulate transcript stability or translation. While it was demonstrated that RNPC1 stabilizes HuR mRNA by binding to its 3' UTR, it is currently unclear whether RNPC1 and HuR bind the same sequences within the 3' UTR[25]. As will be described below, the RBP tristetraprolin (TTP) can also bind to the HuR mRNA to promote its degradation, and the cellular levels of TTP can affect promotion of a tumor phenotype[26]. These studies indicate that regulation of HuR mRNA stability is likely to involve multiple protein binding sites and RBPs that can vary depending on the state of cellular health and growth. Figure 1 summarizes the transcriptional and post-transcriptional mediators that regulate expression of HuR mRNA.

Expression patterns during development, aging, and cellular senescence

HuR was first described as the fourth member of the ELAV family of proteins that was originally identified in Drosophila. The three original members (currently called HuB, HuC, and HuD) were shown to have neuron-specific expression, but unexpectedly, a fourth member (now called HuR) was predicted by polymerase chain reaction and low-stringency cDNA library screening of vertebrates and was expressed in all tissues tested, including brain, kidney, lung, heart, liver, muscle, skin, testis, and ovary[27]. Shortly thereafter, a cDNA was isolated from HeLa cells and the corresponding mRNA was similarly found to be expressed in a wide variety of human tissues[28]. A murine version was described one year later[29]. Assays of mouse tissues from early embryogenesis to adulthood were performed to determine levels of HuR expression during vertebrate development. From the small number of embryonic and extra-embryonic tissues selected for assays, HuR was shown to have developmental age- and tissue-specific variability in expression. Interestingly, HuR levels strongly paralleled levels of AUF1, another RBP that binds AREs in target mRNAs, but promotes their degradation[30,31]. A more comprehensive examination of HuR levels in adult murine tissues showed that HuR protein was most strongly expressed in lymphoid tissues, intestine, and testes, with moderate expression in liver and uterus, and the lowest expression in brain, heart, lung, kidney, skeletal muscle, and ovary[32].

Early studies of HuR expression also examined its levels and effects on cell health during cellular aging. In multiple *in vitro* models of cellular senescence, HuR levels were shown to decrease, as did the half-lives of HuR's corresponding mRNA targets. Further, HuR overexpression and knockdown of expression with antisense RNAs revealed a direct relationship between the levels of HuR and a "younger" cell phenotype[33]. However, a follow-up study examining the levels of mRNA regulatory proteins in human tissue arrays from individuals of various ages, revealed that HuR expression remained relatively unchanged with increasing age[34]. Therefore, the significance of the cellular senescence studies as they relate to human aging is unclear. The human tissue array study also confirmed previous murine studies in demonstrating tissue-specific levels of expression and the parallel expression of HuR and ARE-binding protein AUF1. Other studies, described below, indicate the importance of maintaining appropriate balances of RBPs that both degrade and stabilize ARE-containing mRNAs[26], so the parallel expression of HuR and AUF1 is likely a mechanism to ensure an appropriate balance of these mRNA transcripts.

Regulation of HuR translation and protein stability

In recent years, regulation of HuR biosynthesis by microRNAs (miRNAs) has been identified as a key process in controlling HuR levels. Multiple miRNAs, including miR-16 and miR-519, have been identified as inhibitors of HuR translation via direct binding of HuR mRNA. These miRNAs have been implicated in suppression of tumor cell growth through inhibition of HuR synthesis, and discussion of their function will be addressed below. In this section, we will discuss mechanisms that modulate HuR expression through regulators of protein stability and cleavage.

HuR is subject to multiple levels of post-translational regulation from diverse signals. As stated above, nucleocytoplasmic shuttling is an important mechanism by which HuR can be triggered to exert differential effects in cells. As previously reviewed, phosphorylation by various kinases, including checkpoint kinase 2 (Chk2), Cdk1, p38, and PKC, can regulate HuR levels in the cytoplasm and/or

binding to target mRNAs[35]. HuR methylation by CARM1 (co-activator-associated arginine methyltransferase 1) can similarly affect HuR activity[36]. However, control of HuR protein levels and function through degradation or cleavage also has been shown to be key to HuR's effects on cellular processes. Mild heat shock was demonstrated to rapidly decrease HuR protein without altering mRNA levels or translation rates. This loss of HuR was found to be due to ubiquitin-mediated proteolysis and is believed to enhance cell survival by altering the stability and/or translation of HuR target mRNAs[37]. Through a different pathway, ubiquitin-mediated proteolysis was also shown to cause HuR degradation when cancer cells are subjected to inhibition of glycolysis, which may represent an attempt to slow proliferation in the absence of cellular energy[38].

Post-translational regulation of HuR protein levels can be altered depending on the context of the stress. Mild stresses most often induce translocation of HuR from the nucleus to the cytoplasm, resulting in increased cell survival. It was reported that lethal stress such as treatment with the apoptosis inducer staurosporine also results in HuR translocation to the cytoplasm; however, once there, HuR may be cleaved by caspases, leading to an enhanced apoptotic response[39]. Similar caspase-mediated cleavage events were noted in cells subjected to chronic, but not acute, hypoxia. Further, one of the HuR cleavage products produced was demonstrated to bind the 3' UTR of a HuR target mRNA (c-myc) and block its translation, leading to decreased cell viability[40]. A very recent study has demonstrated that under a lethal stress (staurosporine), HuR, which normally binds and stabilizes both pro- and anti-apoptotic mRNA targets, was cleaved and the resulting cleavage products bound and stabilized the pro-apoptotic mRNA caspase-9, but not the anti-apoptotic target prothymosin[10]. These results all suggest that the cleavage of HuR under lethal stress results in products that shift HuR's function from a pro-survival factor to a pro-apoptosis activator.

PATHOLOGICAL OVEREXPRESSION OF HUR, REGULATION BY MICRO-RNAS, AND CANCER

HuR levels are elevated in numerous types of cancer

The importance of HuR to cell survival and proliferation is made evident by the many types of tissues in which elevated HuR levels are associated with cancer. These tumor types include breast, lung, ovarian and colon cancers[41] and numerous other tissues. Notably, while HuR is typically localized primarily to nuclei, high cytoplasmic levels of HuR are known to be associated with worse prognosis in numerous types of cancers including human lung adenocarcinoma[42], gall bladder carcinoma[43], urothelial carcinoma[44], ovarian cancer[45], breast cancer[46,47], cervical cancer[48], laryngeal squamous cell cancer[49], and colon cancer[50]. HuR has been shown to interact with and regulate a large number of mRNA transcripts with AREs that are involved in oncogenic cellular transformation. As previously reviewed, these include regulators of cell growth and division (*e.g.,* c-myc, cyclins), gene products involved in invasion and metastasis (*e.g.,* MMP-9), pro-survival mediators (*e.g.,* prothymosin-α), and products that can trigger local angiogenesis [*e.g.,* vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1 (HIF-1)][41].

Early work to examine the mechanisms behind regulation of HuR expression in cancer was performed in gastric tumor cells that expressed high levels of HuR. No genetic or epigenetic alterations were noted in these cells, but the elevated HuR expression was found to be dependent on excessive levels of PI3K-Akt signaling. Further, NF-κB, a downstream regulator of Akt, was shown to directly activate HuR transcription through a binding site in the HuR promoter. Akt was also implicated in promoting transport of HuR from the nucleus to the cytoplasm[12]. Although this study clearly implicated aberrant transcriptional control of HuR as contributing to cancer, most of our understanding of HuR's regulation in cancer cells comes from analysis of its interactions with miRNAs. MiRNAs are small (about 22 nucleotides) noncoding RNA molecules that post-transcriptionally regulate gene expression by inducing mRNA degradation and/or suppressing translation. The interplay between HuR and microRNAs is complex, since defined miRNAs directly regulate HuR expression, and HuR is capable of inhibiting miRNA-mediated suppression or activation of target mRNAs. The latter effect occurs mostly in mRNAs that contain AREs downstream of miRNA binding sites in their 3' UTR. It is postulated that miRNA target sites extensively overlap with HuR binding sites that are observed even in the intronic regions of various growth promoting gene transcripts[51], and the relationship between HuR and various miRNAs is usually functionally antagonistic. Competitive interaction usually results in an enhanced gene expression if HuR-mRNA binding dominates. However, when both HuR and miRNAs cooperatively bind transcripts, such mRNAs are usually expressed at lower levels[52]. MicroRNAs are differentially expressed in tumor cells and their interactions with RBPs such as HuR may eventually determine the outcome of tumor progression, chemotherapy and drug resistance. The variation of miRNA expression in primary versus metastatic tumors may partially explain the aggravated tumorigenic response in spite of interventional procedures in a number of cancers[53].

Regulation of HuR expression by microRNAs

The first miRNA demonstrated to regulate HuR was miR-519, as predicted by sequence analysis and confirmed by experimental procedures in 2008[54]. MiR-519 binding sites were identified in both the coding region and 3' UTR of HuR. MiR-519 was shown to inhibit HuR expression in multiple tumor cell lines by suppressing HuR translation, but not HuR mRNA levels. Modulating the levels of miR-519 within cells affected HuR downstream tar-

gets. Not unexpectedly, decreasing the ability of miR-519 to bind HuR (through addition of antisense miR-519), increased HuR levels and the rate of cell division. In a subsequent study, HuR and miR-519 levels were examined in pairs of cancerous and adjacent healthy tissue[55]. HuR protein, but not mRNA, levels were increased in the cancer samples, and miR-519 levels were markedly reduced. miR-519 was also shown to inhibit tumor growth from HeLa cells injected into athymic mice, supporting the notion of miR-519 as a tumor suppressor that acts through HuR[55]. Notably, miR-519 levels were demonstrated to increase in a model of cellular senescence, suggesting that triggering of senescence through inhibition of HuR is a mechanism by which tumor suppression may occur[56].

In the last five years, several new miRNA regulators of HuR have been identified. MiR-16 was demonstrated to translationally repress HuR in breast cancer cells by interacting with the 3' UTR of HuR mRNA[57]. This miRNA also suppresses translation of COX-2, tumor necrosis factor-α and Bcl-2[58,59], which, interestingly, are all tumor-promoting genes positively regulated by HuR. The complexity of the interaction between HuR and miR-16 was demonstrated to an even greater degree when it was shown that association of a HuR/miR-16 complex with AREs of several target transcripts could facilitate inhibition of miR-16 expression in colorectal cancer cells[60]. Thus, the tumor suppressor activity of miR-16 and the tumor-promoting activities of HuR appear to antagonize one another at multiple levels.

miR-125a was first reported to inhibit cell growth and promote apoptosis by translationally repressing HuR in breast cancer cells[61]. In another study, miR-125 was shown to inhibit phosphorylation of Akt in breast cancer cells[62]. This suppression of Akt activation could interfere with the growth-promoting environment through various downstream pathways, one of which is the transcriptional activation of HuR expression through Akt/NF-κB signaling[12,13]. Thus, miR-125 may inhibit HuR expression at multiple levels, through direct translational suppression and through indirect inhibition of transcription. Overexpression of another miRNA, miR-34a, was shown to suppress HuR protein levels in prostate cancer cells, thus modulating cell proliferation and drug resistance in those cells[63]. However, no potential binding sites for miR-34a were found by *in silico* analysis of the HuR 3' UTR, suggesting that miR-34a may regulate HuR through binding in other regions of the transcript or through other mechanisms. MiR-9 similarly acts as a tumor suppressor by directly binding the 3' UTR of HuR, thus suppressing HuR expression and expression of its downstream targets[64]. HuR has also recently been reported to be a target of miR-146, a potent anti-inflammatory molecule[65]. HuR was shown to be a direct target of miR-146, which suppresses both HuR mRNA and protein levels. HuR is established as a regulator of mRNAs involved in inflammation[35,66], as well as a positive regulator of NF-κB activity[13,67]. Thus, one pathway through which miR-146 exerts its anti-inflammatory effects is through suppression of HuR.

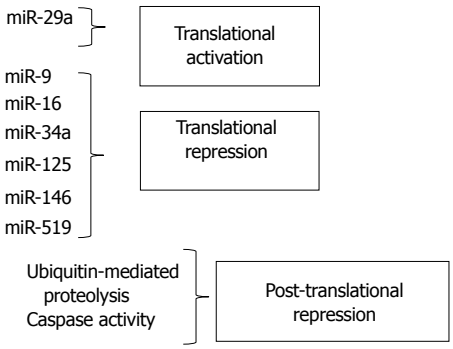

Figure 2 Regulators of HuR mRNA Expression.

While the miRNAs described above all directly bind to HuR mRNA and inhibit synthesis of the protein, other miRNAs can positively regulate HuR synthesis through indirect mechanisms. MiR-29a, a miRNA abundant in breast cancer cells, binds to and degrades the mRNA transcript of tristetraprolin (TTP), another RBP that works to promote decay of target mRNAs. Because HuR mRNA is a target of TTP-mediated degradation, miR-29a's overall effects are to reduce TTP levels while increasing HuR expression. This imbalance in the HuR/TTP ratio correlated with increased expression of ARE-containing, tumor-promoting mRNAs. Importantly, inhibition of miR-29a reversed the imbalance, suggesting this microRNA as a potential target for inhibition in breast cancer[26]. This study demonstrates that miRNA-mediated regulation of other RBPs is critical to the overall activity of HuR and provides insight into ways in which a network of RBPs can control cell fate. Figure 2 summarizes the effects of miRNAs along with post-translational mechanisms in regulating cellular levels of HuR.

SUMMARY AND FUTURE DIRECTIONS

The diverse molecular functions of HuR in both normal and malignant tissues have prompted researchers to probe the role of this master regulator in various types of human cancer, inflammation, and other diseases. Elucidating the mechanisms of transcriptional, post-transcriptional, translational, and post-translational regulation of HuR in both cellular stress and disease can provide critical insights into HuR's overarching control of cellular processes. Although our knowledge of these mechanisms has expanded rapidly over the last decade, there are many aspects of HuR expression that still require study. The full range of transcription factors that regulate HuR expression is not yet elucidated, and how aberrant transcription of HuR might lead to cancer and other diseases is largely unexplored. Further, the functional relevance of HuR mRNAs with alternate 5' and 3' UTRs is still not well developed. Multiple studies, including our own, have noted uncoupling of HuR mRNA and protein levels in both normal and malignant tissues[29,66,68], and the mechanisms behind this uncoupling must still be investigated. However, this phenomenon is likely to be related, at least

in part, to the presence of alternate mRNA HuR transcripts with different translatabilities and mRNA half-lives. How these alternate mRNAs are generated and translated during normal cell growth, cellular stress, and oncogenesis must still be determined. It is clear that HuR itself is likely to be subjected to post-transcriptional regulation by a variety of RBPs that are still undefined.

Increasing evidence demonstrates that miRNAs are involved in a complex, intricate network with HuR to post-transcriptionally regulate genes involved in development, stress, cell cycle, and cell survival, and this interplay might very well regulate a multitude of disease pathways. Fine tuning of such regulatory networks between a large repertoire of miRNAs and RBPs very well might be a trigger or a switch dictating the fate of every cell in the human body. Although it is clear from murine knockout studies that HuR expression is critical for organismal development and survival[69], little is known about how differences in HuR expression among various tissues and at different stages of development affect these processes.

In this review, we have used HuR's role in cancer to illustrate how alteration of its expression can contribute to human disease. MiRNA and HuR interactions are in the limelight for their outcomes in the field of cancer, but it is also clear that alterations in HuR expression are associated with other physiological and pathological processes. For example, expression of HuR mRNA, as well as two of its targets, the VEGF and HIF-1α mRNAs, was shown to increase in animal models of hypoxia[70]. Subsequently, we demonstrated increased levels of HuR protein expression in rat kidneys subjected to ischemia-reperfusion injury, and only in the regions of the kidney susceptible to damage[14]. Heightened HuR levels have also been associated with a number of vascular pathologies[71]. However, the mechanisms behind these changes in HuR levels have not been determined. Through *in vitro* studies of normal, stressed, and cancer cells, there now exists a catalogue of mechanisms by which HuR expression is regulated. These findings should provide a strong basis for understanding the molecular changes that result in altered HuR expression in other diseases. Work to extend these findings to physiological and disease processes at the whole animal level should now be undertaken.

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P- Reviewers: Demonacos C, Teng RJ **S- Editor**: Zhai HH
L- Editor: A **E- Editor**: Yan JL

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Online Submissions: http://www.wjgnet.com/esps/
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doi:10.4331/wjbc.v4.i4.119

World J Biol Chem 2013 November 26; 4(4): 119-130
ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Effect of alcohol exposure on hepatic superoxide generation and hepcidin expression

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Supported by Grant to Harrison-Findik DD, No. NIH R01AA017738; University of Nebraska Medical Center Undergraduate Scholarship to Lu S EPR spectroscopy studies were conducted in the EPR Core Facility, which is supported by the University of Nebraska-Lincoln Redox Biology Center, No. NIH 1 P30 GM103335
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Received: August 21, 2013 Revised: November 5, 2013
Accepted: November 15, 2013
Published online: November 26, 2013

Abstract

AIM: To understand the role of mitochondrial-produced superoxide ($O_2^{\bullet-}$) in the regulation of iron-regulatory hormone, hepcidin by alcohol in the liver.

METHODS: For alcohol experiments, manganese superoxide dismutase knockout mice heterozygous for *Sod2* gene expression (*Sod2*$^{+/-}$) and age-matched littermate control mice (LMC), expressing *Sod2* gene on both alleles, were exposed to either 10% (w/v) ethanol in the drinking water or plain water (control) for 7 d. Total cellular $O_2^{\bullet-}$ levels in hepatocytes isolated from the livers of mice were measured by electron paramagnetic resonance spectroscopy. The mitochondrial-targeted, $O_2^{\bullet-}$-sensitive fluorogenic probe, MitoSOX Red and flow cytometry were utilized to measure $O_2^{\bullet-}$ in mitochondria. Gene and protein expression were determined by Taqman Real-time quantitative PCR and Western blotting, respectively.

RESULTS: *Sod2*$^{+/-}$ mice expressed 40% less MnSOD protein (SOD2) in hepatocytes compared to LMC mice. The deletion of *Sod2* allele did not alter the basal expression level of hepcidin in the liver. 10% ethanol exposure for 1 wk inhibited hepatic hepcidin mRNA expression three-fold both in *Sod2*$^{+/-}$ and LMC mice. $O_2^{\bullet-}$ levels in hepatocytes of untreated *Sod2*$^{+/-}$ mice were three-fold higher than in untreated LMC mice, as observed by electron paramagnetic resonance spectroscopy. $O_2^{\bullet-}$ levels in mitochondria of *Sod2*$^{+/}$ mice were four-fold higher than in mitochondria of untreated LMC mice, as measured by MitoSOX Red fluorescence and flow cytometry. Alcohol induced a two-fold higher increase in $O_2^{\bullet-}$ levels in hepatocytes of LMC mice than in *Sod2*$^{+/-}$ mice compared to respective untreated counterparts. In contrast, 1 wk alcohol exposure did not alter mitochondrial $O_2^{\bullet-}$ levels in both *Sod2*$^{+/-}$ and control mice.

CONCLUSION: Mitochondrial $O_2^{\bullet-}$ is not involved in the inhibition of liver hepcidin transcription and thereby regulation of iron metabolism by alcohol. These findings also suggest that short-term alcohol consumption significantly elevates $O_2^{\bullet-}$ levels in hepatocytes, which appears not to originate from mitochondria.

Key words: Hepcidin; Alcohol; Iron; Superoxide; Superoxide dismutase; Liver; Mitochondria; Manganese superoxide dismutase; Electron paramagnetic resonance

Core tip: Patients with alcoholic liver disease frequently exhibit iron overload, which contributes to liver injury. Our previous research has shown that suppression of

iron-regulatory hormone, hepcidin by alcohol-mediated oxidative stress in the liver is involved. This manuscript investigates the role of superoxide and mitochondria in this process by using MnSOD knockout mice.

Harrison-Findik DD, Lu S, Zmijewski EM, Jones J, Zimmerman MC. Effect of alcohol exposure on hepatic superoxide generation and hepcidin expression. *World J Biol Chem* 2013; 4(4): 119-130 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i4/119.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i4.119

INTRODUCTION

Alcoholic liver disease (ALD) patients frequently display evidence of iron overload[1-4]. Even moderate alcohol consumption can elevate serum iron indices[5]. Both iron and alcohol individually cause oxidative stress, which culminate in liver injury[6]. Hepcidin is an antimicrobial peptide, which is synthesized in the hepatocytes of the liver[7,8]. It plays a central role in the regulation of iron metabolism by inhibiting intestinal iron transport and the release of iron from macrophages[9,10]. Hepcidin achieves this by binding to the iron exporter ferroportin and inducing its internalization and degradation[11]. We have previously demonstrated that short-term and moderate alcohol exposure is sufficient to suppress hepcidin synthesis in the liver, which in turn causes an increase in the expression of intestinal iron transporters *in vivo*[12]. Vitamin E and N-acetylcysteine (NAC) administration *in vivo* abolishes the effect of alcohol on both liver hepcidin and duodenal iron transporters[12]. Vitamin E is a peroxyl radical scavenger and inhibits lipid peroxidation of cellular membranes[13]. NAC can either directly interact with reactive oxygen species (ROS) *via* its free thiol group or exert an indirect antioxidant effect as a precursor of reduced glutathione (GSH)[14,15]. Our findings therefore suggest that early changes in free radical scavenging and antioxidant defense mechanisms induced by alcohol are involved in the inhibition of hepcidin expression and alcohol-induced accumulation of iron.

Superoxide ($O_2^{\bullet-}$) can initiate lipid peroxidation, and iron can interact with $O_2^{\bullet-}$ and hydrogen peroxide to yield the highly reactive hydroxyl radical[16,17]. The antioxidant enzyme, superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\bullet-}$ into hydrogen peroxide and oxygen[18-20]. Mammalian cells express three types of SOD with different subcellular localization and metal requirements. CuZnSOD (SOD1) is primarily localized in the cytoplasm, but is also found in mitochondria and the nucleus. MnSOD (SOD2) is strictly targeted to the mitochondrial matrix and SOD3 (EC-SOD) is secreted into the extracellular space. The effect of short-term alcohol intake on SOD expression and $O_2^{\bullet-}$ production in the liver is unclear. Chronic alcohol consumption however has been shown to elevate SOD2 expression in livers of monkeys and in sera of ALD patients[21,22]. Another study with liver biopsies from ALD patients reported a decrease in SOD1 expression, which correlated with liver fibrosis[23]. Rats with chronic ethanol ingestion also exhibited a decrease in liver SOD1 activity[24]. Delivery of adenoviral SOD1 into rats with chronic alcohol exposure has been reported to reduce alcohol-induced liver injury[25]. Kessova *et al*[26] reported necrosis, inflammation and apoptosis in the livers of knockout mice homozygous for *Sod1* (*Sod1*$^{-/-}$) following alcohol exposure. In patients with ALD or in other experimental models of ALD, alcohol induces lipid accumulation and elevates GSH in the liver[27,28]. In contrast, *Sod1*$^{-/-}$ mice exhibited milder lipid accumulation and decreased GSH in the liver compared to alcohol-fed wildtype control mice[26]. Curry-McCoy *et al*[29] also reported atypical responses to chronic alcohol consumption in *Sod1*$^{-/-}$ mice.

Mitochondria play a key role in iron biogenesis, and alcohol metabolism[30]. In mitochondria, $O_2^{\bullet-}$ is mainly produced from complex I (NADH-ubiquinone oxidoreductase) and complex Ⅲ (ubiquinol-cytochrome oxidoreductase) of the electron transport chain. Both complex I and complex Ⅲ have been shown to be involved in alcohol-mediated ROS production[31]. Separate clinical studies with French alcoholic cirrhosis patients suggested that a genetic polymorphism in the mitochondrial targeting sequence of SOD2, which enhances the mitochondrial import of SOD2, increases the risk for iron accumulation in hepatocytes and hepatocellular carcinoma[32,33]. However, another independent study with ALD patients did not find an association between SOD2 genetic polymorphisms and alcohol-induced oxidative stress or liver fibrosis[34]. Over expression of SOD2 by recombinant adenovirus has been reported to prevent liver injury induced by intragastric chronic alcohol feeding in rats[35]. In contrast, another study with mice has shown that elevated SOD2 expression worsens the effect of prolonged alcohol consumption but is protective when mice receive a single intragastric dose of alcohol (*i.e.,* an alcohol binge)[36]. Larosche *et al*[37] have reported that an alcohol binge further decreases SOD2 activity in mice lacking one *Sod2* allele but further increases it in mice overexpressing SOD2.

The effect of moderate short-term alcohol exposure on SOD2 expression and $O_2^{\bullet-}$ generation, and the role of $O_2^{\bullet-}$ in the regulation of hepcidin transcription in the liver requires further investigation. This study aims to address these questions by using knockout mice heterozygous for *Sod2* and control mice as a model.

MATERIALS AND METHODS

Animal experiments

Animal experiments were approved by the Institutional Animal Care and Use committee at the University of Nebraska Medical Center. Manganese superoxide dismutase (MnSOD) knockout mice lacking the expression of *Sod2* gene, on a C57BL/6 genetic background, were generated, as described[38]. We employed knockout mice heterozy-

gous for *Sod2* gene expression ($Sod2^{+/}$) and age-matched littermate control mice (LMC) expressing *Sod2* gene on both alleles. All mice were maintained on a regular chow diet (Harlan Teklad 7012). For alcohol experiments, 6-8 wk old male mice were housed in individual cages and exposed to either 10% (w/v) ethanol in the drinking water or plain water (control) for 7 d, as described previously[12,39], at which time livers were collected. Before harvesting, livers were perfused with warm phosphate-buffered saline (PBS) buffer (pH 7.4) to eliminate blood. Livers isolated from mice were snap frozen in liquid nitrogen and stored at -80 ℃ until further use. For electron paramagnetic resonance (EPR) spectroscopy, fresh livers were subjected to a standard perfusion protocol to isolate viable hepatocytes, as described below.

Liver perfusion

To isolate viable hepatocytes, mice livers were perfused, as described[40]. Briefly, livers were perfused (7 mL/min) *via* the inferior vena cava with warm and gassed KRH buffer (250 mmol/L HEPES, 1149 mmol/L NaCl, 45 mmol/L KCl, 10 mmol/L KH_2PO_4, 0.5 mmol/L EGTA, pH 7.6) followed by KRH buffer containing 2 mmol/L Ca^{2+} and collagenase (0.214 mg/mL, Sigma C5138). Hepatocytes, isolated by centrifugation (50 *g*, 2 min), were washed thrice with ice-cold KRH buffer containing 2 mmol/L Ca^{2+} and 2% BSA. Dead hepatocytes were discarded by Percoll centrifugation (129 *g*, 5 min 4 ℃) and cell viability was determined by Trypan Blue staining. Hepatocytes with ⩾ 78% viability were employed for experiments.

Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance (EPR) was used to measure total cellular $O_2^{\bullet-}$ levels in hepatocytes isolated from the livers of $Sod2^{+/-}$ and control littermate mice fed either with plain (control) or ethanol-supplemented water, as described above. 0.5×10^6 cells were incubated with the cell permeable and $O_2^{\bullet-}$ sensitive spin probe, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, 200 μmol/L) for 15 min at 37 ℃ in EPR buffer (99 mmol/L NaCl, 4.69 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 1.03 mmol/L KH_2PO_4, 5.6 mmol/L D(+)-glucose, 20 mmol/L Na-Hepes, pH 7.4) freshly supplemented with metal chelators, 5 μmol/L diethyldithiocarbamic (DETC) acid (sodium salt) and 25 μmol/L deferoxamine methanesulfonate. 50 μL of cell suspension was loaded into a glass capillary tube, which was then inserted into the capillary holder of a Bruker e-scan EPR spectrometer. The following EPR settings were used for all experiments: field sweep width, 60 G; microwave frequency, 9.75 kHz; microwave power, 21.90 mW; modulation amplitude, 2.37 G; conversion time, 10.24 ms; time constant, 40.96 ms. The EPR amplitude, which is directly proportional to the levels of $O_2^{\bullet-}$ in the sample, were normalized to the number of hepatocytes in each sample.

Flow cytometry with MitoSOX

MitoSOX Red fluorescence was used to measure mitochondrial-localized $O_2^{\bullet-}$ levels in hepatocytes isolated from the livers of $Sod2^{+/-}$ and control littermate mice fed either with plain water (control) or ethanol, as described above. 0.5×10^6 viable cells were incubated with 2 μmol/L MitoSOX™Red dye (Invitrogen) or buffer for 10 min in a 37 ℃ shaker in the dark. Samples were washed to eliminate free dye and MitoSOX Red fluorescence was collected using a FACSCalibur flow cytometer and analyzed with Cell Quest Pro (BD Biosciences, San Jose, CA, United States). Standard instrument configuration was used as follows: excitation at 488 nmol/L and the fluorescence collected at 585/42 (FL2) and 670LP (FL3) channel. Analysis of the FL2 signal is presented here and data are expressed as mean channel number compared to an unstained (buffer control) sample.

RNA isolation, cDNA synthesis, real-time quantitative polymerase chain reaction, and reverse transcription-polymerase chain reaction analysis

RNA isolation, cDNA synthesis and real-time quantitative polymerase chain reaction (PCR) were performed, as published previously[12,41]. Primers (sense 5'-ACTCGGACCCAGGCTGC-3'; antisense 5'-AGATAGGTGGTGCTGCTCAGG-3') and Taqman fluorescent probe [5' 6-(FAM)-TGTCTCCTGCTTCTCCTCCTTGCCA-3' (TAMRA-Q)] flanking about 70 base pairs of open reading frame sequences of mouse hepcidin genes, *Hamp1* and *Hamp2* were designed by the Primer Express 1.5 program (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (gapdh) gene probe was used as the endogenous control. For reverse transcription-polymerase chain reaction (RT-PCR), cDNA was amplified using primers for *Sod1* (sense 5'-ATGGCGATGAAAGCGGTGT-3'; antisense 5'-CCTTGTGTATTGTCCCCATACTG-3'), *Sod2* (sense 5'-CAGACCTGCCTTACGACTATGG-3'; antisense 5'-CTCGGTGGCGTTGAGATTGTT-3'), or gapdh (sense 5'-GTGGAGATTGTTGCCATCAACGA-3'; antisense 5'-CCCATTCTCGGCCTTGACTGT-3') by Taq polymerase for 23 cycles (95 ℃ for 1 min, 60 ℃ for 30 s, 72 ℃ for 30 s and a final extension at 72 ℃ for 5 min) following the initial denaturation step at 95 ℃ for 5 min. PCR products separated on 2% agarose gel were stained with ethidium bromide and visualized under UV transillumination.

Mitochondria and cell lysate preparation, and western blotting

To isolate mitochondria-enriched fractions, mice livers were homogenized in ice-cold mitochondria buffer [20 mmol/L Hepes, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L DTT, 250 mmol/L Sucrose, 0.1 mmol/L PMSF, pepstain A, protease inhibitor cocktail (Sigma P2714), pH 7.5] and centrifuged at 800 *g* for 10 min at 4 ℃. The resultant supernatant was centrifuged at 16000 *g* for 20 min at 4 ℃. The pellet was collected and washed twice in ice-cold mitochondria buffer by centrifugation (16000 *g* for 20 min). The washed pellet fraction was subsequently lysed in Buffer B [10 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 0.5% Triton X-100, 0.1 mmol/L PMSF, pepstain A, protease in-

hibitor cocktail (Sigma P2714), pH 8.0] on ice for 10 min The lysates were centrifuged briefly (100 *g*, 3 min, 4 ℃) to discard cellular debris and the supernatants were kept frozen until further use. To isolate total cell lysates, mice livers were homogenized in a cell lysis buffer [10 mmol/L Tris-HCl, 100 mmol/L NaCl, 5 mmol/L EDTA, 10% glycerol, 0.1 mmol/L PMSF, pepstain A, protease inhibitor cocktail (Sigma P2714), phosphatase inhibitor cocktail A (Santa Cruz, sc-45044), 1% Triton-X-100, (pH 7.4)]. The homogenates were subsequently incubated on ice for 20 min and centrifuged (3000 *g*) for 5 min at 4 ℃. Supernatants were employed for western blotting. Anti-Mn-SOD (SOD2), anti-CuZnSOD (SOD1), anti-cytochrome C, anti-COX4, and anti-gapdh antibodies were obtained commercially (Santa Cruz). Standard Western blots analysis was performed, as described previously[41,42].

Caspase-3 assay

Mice livers were homogenized in lysis buffer (20 mmol/L KCl, 20 mmol/L MOPS, 2 mmol/L $MgCl_2$, 1 mmol/L EDTA, 0.5% Triton X-100, pH 7.2) and incubated on ice for 30 min. Following centrifugation (14500 *g*, 30 min, 4 ℃), the supernatants were collected and used for the assay. Caspase-3 enzyme activity was measured by using Ac-DEVD-AMC caspase-3 fluorogenic substrate (BD Biosciences) and quantifying the amount of AMC released with a Perkin-Elmer Luminescence Spectrophotometer LS 55. Commercially obtained free AMC (Sigma) was used to create a standard curve. Caspase-3 enzyme activity is expressed as nmoles of AMC released per mg of protein. The protein concentrations in liver lysates were determined by the Bradford protein assay.

Statistical analysis

Statistical analysis of differences in treatment groups was performed using the parametric Anova, non-parametric Mann-Whitney tests, and unpaired two-tailed Student's *t* test. *P* value less than 0.05 was considered statistically significant.

RESULTS

Hepatic Sod1 and Sod2 mRNA expression in MnSOD (Sod2) knockout mice

We have previously shown the involvement of acute alcohol-induced oxidative stress in the regulation of hepcidin transcription in the liver *in vivo*[12]. In order to study the effect of mitochondrial-produced $O_2^{\cdot-}$ in this process, we employed *Sod2* transgenic mice (see methods). Knockout mice homozygous for *Sod2* (*Sod2*$^{-/-}$) are not viable while heterozygous (*Sod2*$^{+/-}$) mice, which express 48%-55% of mitochondrial MnSOD activity, are viable[38]. *Sod2*$^{+/-}$ mice expressing both mutant and wildtype *Sod2* alleles, and littermate control (LMC) mice expressing only wildtype *Sod2* allele were identified by genotyping, as described (Figure 1A)[38]. To determine the hepatic *Sod1* and *Sod2* gene expression levels, hepatocytes from the livers of *Sod2*$^{+/-}$ and LMC mice were isolated by a standard perfusion protocol, as described in Materials and Methods.

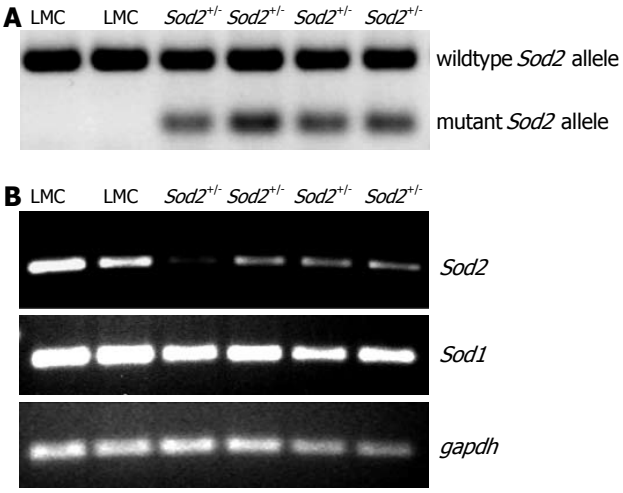

Figure 1 Genotyping and expression. A: Genotyping of mice. Genomic DNA, isolated from the tails of mice by using a commercial kit (Promega), was employed to amplify wild type and mutant *Sod2* allele by PCR, as described[38]. Heterozygous (*Sod2*$^{+/-}$) mice were expressing both mutant (197 bp) and wildtype (457 bp) *Sod2* alleles whereas littermate control (LMC) mice were expressing only wildtype (457 bp) *Sod2* allele, as shown by the agarose gel stained by ethidium bromide; B: Hepatic *Sod1* and *Sod2* mRNA expression in *Sod2*$^{+/-}$ and LMC mice were determined by RT-PCR, as described in Methods. Gapdh was used as the endogenous control gene. PCR: Polymerase chain reaction; RT-PCR: Reverse transcription-polymerase chain reaction.

The expression level of *Sod2* mRNA, in *Sod2*$^{+/-}$ mice was significantly lower than that in LMC (control) mice, as determined by RT-PCR (Figure 1B). In contrast, the mRNA expression level of *Sod1* in *Sod2*$^{+/-}$ mice was similar to that in LMC mice (Figure 1B). RT-PCR analysis with the whole liver tissues isolated from *Sod2*$^{+/-}$ and LMC mice yielded similar results (data not shown).

Effect of alcohol and $O_2^{\cdot-}$ on hepcidin expression in the liver

For alcohol studies, *Sod2*$^{+/-}$ and LMC mice were administered 10% ethanol in the drinking water or plain water (as control) for 1 wk, as described in Materials and Methods. Short-term alcohol exposure did not induce any lipid accumulation or other histological changes in the livers of both *Sod2*$^{+/-}$ and LMC mice, as determined by HE staining of liver sections (Figure 2). Real-time quantitative PCR experiments were performed to determine the expression of hepcidin mRNA in the livers of control and alcohol-fed *Sod2*$^{+/-}$ and LMC mice. In alcohol-fed LMC mice, hepcidin mRNA expression was significantly inhibited three-fold (0.313 ± 0.144), compared to water-fed LMC mice (Figure 3). As compared to untreated LMC mice, basal hepcidin expression in the livers of *Sod2*$^{+/-}$ mice (1.05 ± 0.276) was unchanged (Figure 3). Alcohol also decreased hepcidin mRNA expression in *Sod2*$^{+/-}$ mice three-fold (0.297 ± 0.121), compared to water-fed LMC and *Sod2*$^{+/-}$ mice (Figure 3).

Short-term alcohol exposure and $O_2^{\cdot-}$ levels in hepatocytes

Hepcidin is primarily synthesized in hepatocytes of the

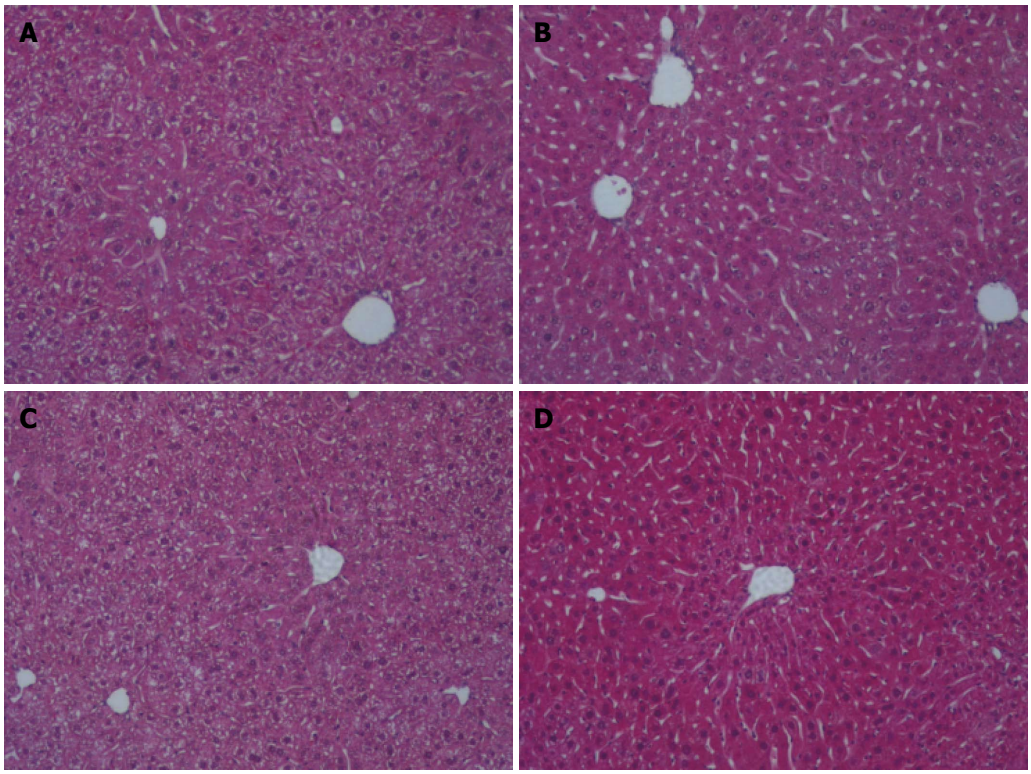

Figure 2 Liver histology. Fixed liver sections of littermate control (LMC) and heterozygous (*Sod2*$^{+/-}$) mice fed with plain water (H_2O) or 10% ethanol for 1 wk were stained with hematoxylin and eosin (original magnification ×10). A: LMC H_2O; B: LMC ethanol; C: *Sod2*$^{+/-}$ H_2O; D: *Sod2*$^{+/-}$ ethanol.

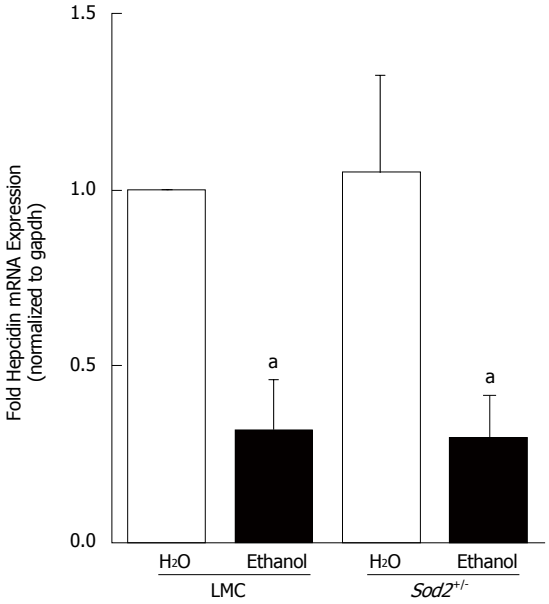

Figure 3 Alcohol and hepcidin mRNA expression. cDNA, synthesized from RNA isolated from the livers of *Sod2*$^{+/-}$ and littermate control mice (LMC) mice fed with 10% ethanol or plain water (H_2O) for one week, was used as a template for real-time quantitative polymerase chain reaction. Hepcidin mRNA expression in alcohol-treated LMC and *Sod2*$^{+/-}$ mice was expressed as fold expression of that in water-treated LMC mice. The letter a indicates statistical significance ($^aP < 0.05$).

liver[7]. In order to study the effect of acute alcohol exposure on $O_2^{\bullet-}$ levels, hepatocytes from the livers of in *Sod2*$^{+/-}$ and LMC mice were isolated. Superoxide levels were quantified by EPR spectroscopy, as described in materials and methods. Levels of $O_2^{\bullet-}$ in untreated *Sod2*$^{+/-}$ mice (1.2×10^6 arbitrary units) were elevated three-fold as compared to untreated LMC mice (0.46×10^6 arbitrary units) (Figure 4A, C and E). Alcohol treatment for 1 wk was sufficient to significantly elevate $O_2^{\bullet-}$ levels in hepatocytes collected from both *Sod2*$^{+/-}$ (2.24×10^6 arbitrary units) and LMC (1.62×10^6 arbitrary units) mice (Figure 4B, D and E). Alcohol intake induced a four-fold (3.63 ± 1.1) increase in the levels of $O_2^{\bullet-}$ in LMC mice as compared to untreated LMC mice. The levels of $O_2^{\bullet-}$ in alcohol-fed *Sod2*$^{+/-}$ mice were two-fold (2.06 ± 0.33) higher than in untreated *Sod2*$^{+/-}$ mice (Figure 4E).

To specifically measure $O_2^{\bullet-}$ in mitochondria, the mitochondrial-targeted, $O_2^{\bullet-}$-sensitive fluorogenic probe, MitoSOX Red and flow cytometry were utilized. FACS analysis histograms and quantification of mean fluorescent intensities show that alcohol did not significantly alter fluorescent intensity of MitoSOX in hepatocytes of alcohol-treated LMC mice compared to untreated LMC mice fed with plain water (Figure 5A-D and I). The deletion of one allele of *Sod2* gene elevated MitoSOX fluorescence four-fold in untreated *Sod2*$^{+/-}$ mice (3.967 ± 0.71) compared to untreated LMC control mice (1 ± 0) (Figure 5E, F and I), thus, indicating an increase in basal mitochondrial $O_2^{\bullet-}$ levels. Similar to LMC mice, 1 wk long alcohol intake did not further increase MitoSOX fluorescence in hepatocytes of *Sod2*$^{+/-}$ mice (Figure 5E-I).

Alcohol and SOD protein expression

In order to determine the effect of alcohol on MnSOD

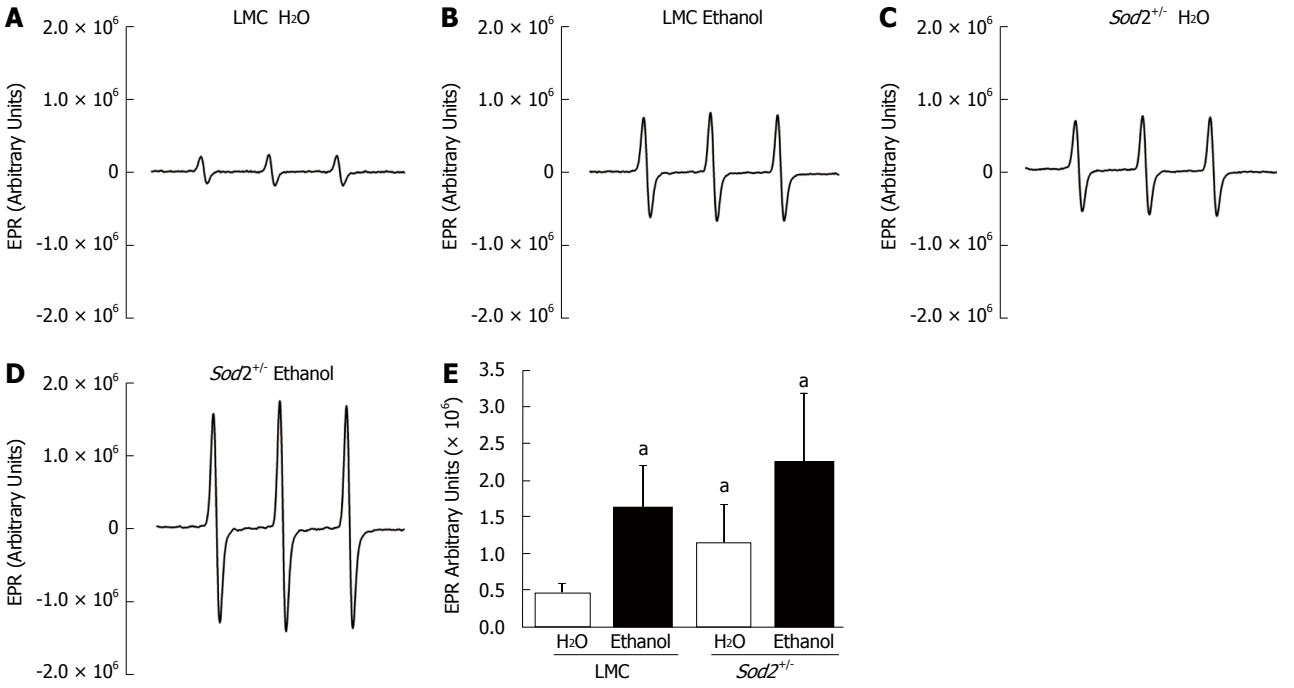

Figure 4 Effect of alcohol on superoxide levels. Hepatocytes, isolated by perfusion from the livers of littermate control (LMC) and *Sod2*$^{+/-}$ mice fed with 10% ethanol or plain water (H_2O), were subjected to electron paramagnetic resonance (EPR) spectroscopy, as described in Methods. A-D: Representative EPR spectra; E: Summary of data presented as mean ± SD, $n = 6$. The letter a indicates statistical significance ($^aP < 0.05$).

protein (SOD2) expression, we performed western blot analysis with whole cell lysates isolated from the hepatocytes of untreated or alcohol-fed *Sod2*$^{+/-}$ and LMC mice livers, as described in Materials and Methods (Figure 6A). The level of SOD2 expression in hepatocytes of *Sod2*$^{+/-}$ mice was 40% lower than that in LMC mice (Figure 6A and B). One week long alcohol exposure did not alter SOD2 protein expression in hepatocytes of LMC mice (Figure 6A and B). Alcohol induced an increase in SOD2 protein expression in *Sod2*$^{+/-}$ mice, which was, however, not significant (Figure 6). Hepatic *Sod2* mRNA levels were also not significantly altered by alcohol exposure in LMC or *Sod2*$^{+/-}$ mice, as determined by real-time quantitative PCR (data not shown). CuZnSOD protein (SOD1) is mainly expressed in the cytosol and chronic alcohol consumption has been suggested to decrease SOD1 protein expression[23,24]. Western blot analysis revealed that short-term alcohol administration does not have an effect on SOD1 protein expression in hepatocytes of both *Sod2*$^{+/-}$ and LMC mice (Figure 7).

The role of alcohol and $O_2^{\bullet-}$ in apoptosis in the liver

Mitochondria and $O_2^{\bullet-}$ are known to play a role in cell death. We therefore examined levels of apoptosis in livers from untreated and alcohol-treated *Sod2*$^{+/-}$ and LMC mice by measuring cytochrome C release from mitochondria and caspase-3 enzyme activity, as described in Materials and Methods. Liver mitochondria and cytosolic fractions isolated from *Sod2*$^{+/-}$ and LMC mice were employed to measure cytochrome C release by western blotting. Levels of cytochrome C protein in mitochondria from untreated and alcohol-treated LMC mice were similar (Figure 8A). The deletion of *Sod2* gene or administration of alcohol did not induce cytochrome C release from mitochondria in *Sod2*$^{+/-}$ mice livers (Figure 8A). A negligible amount of cytochrome C protein was detected in cytosolic fractions, which was similar in all the samples (Figure 8B). The deletion of *Sod2* gene on one allele and $O_2^{\bullet-}$ accumulation in mitochondria did not significantly induce caspase-3 enzyme activity in the livers of *Sod2*$^{+/-}$ mice, compared to untreated LMC mice (Figure 8C). Similarly, 1 wk long alcohol exposure did not significantly activate caspase-3 enzyme in the livers of LMC or *Sod2*$^{+/-}$ mice (Figure 8C). However, the livers of C57BL/6 wildtype mice injected with Fas-agonist antibody, Jo2, which were used as internal control, exhibited a significant increase in caspase 3 activity (Figure 8C).

DISCUSSION

The liver is important for both iron and alcohol metabolism. It is the main site of synthesis for the key iron-regulatory hormone, hepcidin[7]. Transgenic mice deficient in hepcidin expression exhibit severe iron overload in the liver and other organs[43]. The oxidation of ethanol by alcohol dehydrogenase following alcohol consumption occurs in the liver. Alcohol and iron metabolism generate free radicals and lipid peroxidation products[28]. Similar to patients with alcoholic liver disease (ALD), mice with short-term alcohol exposure exhibit reduced hepcidin expression and increased intestinal iron absorption[12,44-46]. We have previously reported that the inhibition of the activity of transcription factor, C/EBPα in the liver is one of the mechanisms involved in the suppression of hepci-

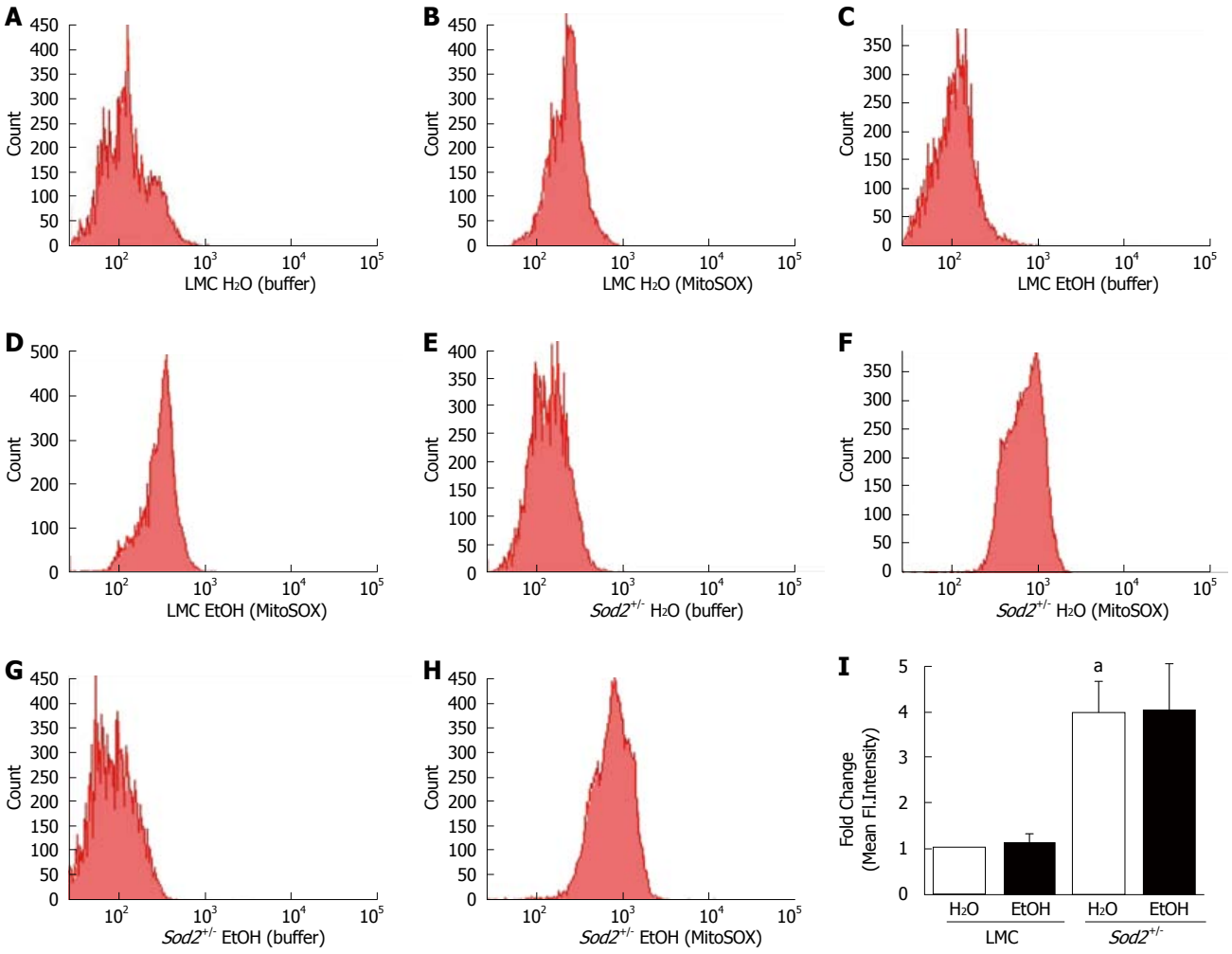

Figure 5 Alcohol and mitochondrial superoxide production. Hepatocytes, isolated from the livers of LMC (A-D) and *Sod2*$^{+/-}$ (E-H) mice fed with plain water (H_2O; A, B, E, F) or 10% ethanol (EtOH; C, D, G, H), were incubated with buffer, as control (A, C, E, G) or MitoSOX Red (B, D, F, H) and analyzed by flow cytometry, as described in Methods. A-H: Representative histograms; I: Quantitative analysis showing fold changes in mean fluorescent intensity in hepatocytes of water-fed *Sod2*$^{+/-}$, and alcohol-treated LMC and *Sod2*$^{+/-}$ compared to that of littermate control (LMC) mice fed with plain water. Samples incubated with MitoSOX Red were normalized for background fluorescence by subtracting the values of control (buffer only) incubations. The letter a indicates statistical significance ($^aP < 0.05$).

din transcription by alcohol[12,39]. We have also shown that 1 wk long alcohol exposure is sufficient to cause oxidative stress in the liver[12]. The administration of antioxidants, vitamin E or NAC reversed the effect of alcohol on the inhibition of both C/EBPα DNA-binding activity and hepcidin expression in the liver as well as on the induction of the intestinal iron transporter expression[12]. The current study investigates the role of $O_2^{\bullet-}$ in the inhibition of hepcidin transcription by alcohol.

Mitochondria are important for iron homeostasis and are involved the pathogenesis of ALD. Alcohol consumption has been shown to increase ROS generation by liver mitochondria[30,47,48]. Considering mitochondria are a primary source of $O_2^{\bullet-}$ production in cells, we employed knockout mice deficient in the expression of SOD2, a mitochondrial-targeted $O_2^{\bullet-}$ scavenging enzyme for these studies. A 1 wk long alcohol exposure model was chosen to study the regulation of hepcidin by mitochondrial $O_2^{\bullet-}$ for the following reasons: (1) The disturbances in the redox balance of the cell are one of the early events in the progression of ALD; (2) We have shown that this model causes oxidative stress and inhibits hepcidin expression in the absence of any lipid accumulation, inflammation or injury in the liver[12]; and (3) The effects of short-term alcohol consumption on $O_2^{\bullet-}$ levels in the liver are unclear. Our previously published *in vivo* studies and other *in vitro* studies employed dihydroethidium staining or ROS-sensitive probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) to evaluate ROS production in the liver induced by short-term alcohol administration[12,48]. In this study, we quantified both total and mitochondrial $O_2^{\bullet-}$ levels by EPR analysis, and MitoSOX Red and flow cytometry, respectively. For these studies, hepatocytes freshly isolated from the livers of alcohol-treated and control mice were employed because hepcidin is primarily synthesized in hepatocytes of the liver.

The deletion of one *Sod2* allele was sufficient to elevate both total and mitochondrial $O_2^{\bullet-}$ levels in hepatocytes by three-fold and four-fold, respectively. In addition, the administration of alcohol for one week caused a significant increase in the level of total $O_2^{\bullet-}$ in both *Sod2*$^{+/-}$ and LMC mice. When compared to untreated counter-

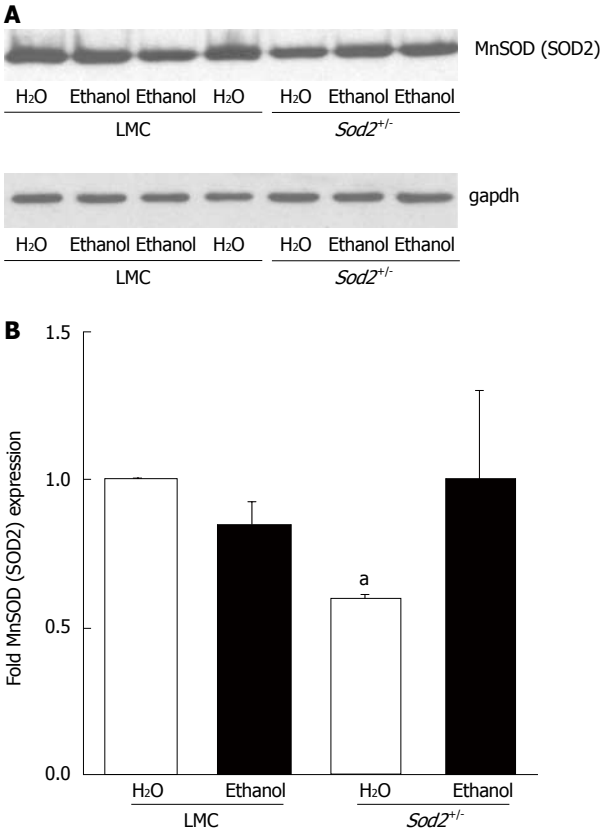

Figure 6 Manganese superoxide dismutase (SOD2**) expression.** A: Whole cell lysate proteins, isolated from the hepatocytes of *Sod2*$^{+/-}$ and littermate control mice (LMC) mice livers treated with 10% ethanol or plain water (H_2O) for 1 wk, were resolved by SDS-Polyacrylamide gel electrophoresis and subjected to western blotting by using an antibody against mouse SOD2. An anti-gapdh antibody was used as control to confirm equal protein loading; B: Autoradiographs were scanned, and SOD2 expression was quantified by normalizing to gapdh protein expression. Normalized protein expression in water-fed *Sod2*$^{+/-}$ and alcohol-fed *Sod2*$^{+/-}$ or littermate control mice was expressed as fold expression of that in water-fed littermate control mice. The letter a indicates statistical significance ($^aP < 0.05$).

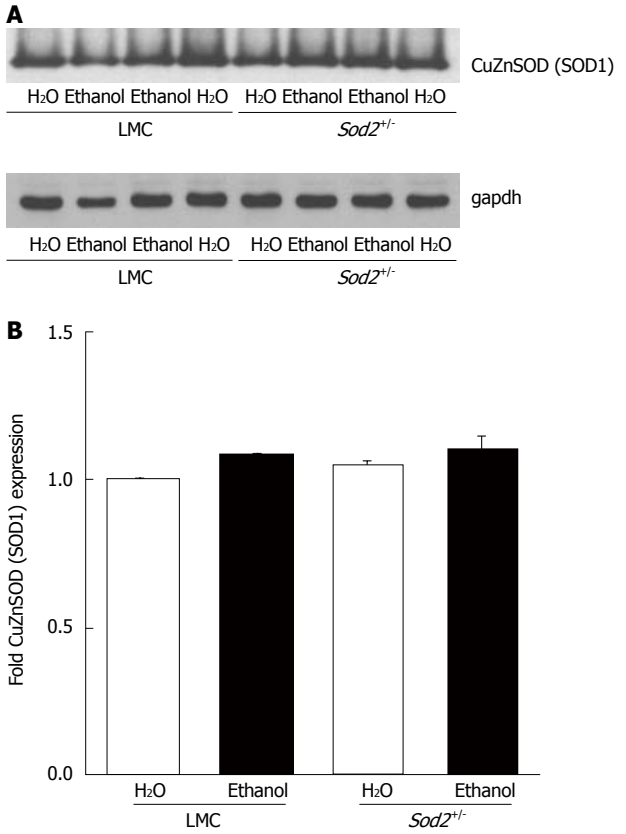

Figure 7 CuZn superoxide dismutase (SOD1) expression. A: Whole cell lysate proteins, isolated from the hepatocytes of *Sod2*$^{+/-}$ and littermate control mice livers treated with 10% ethanol or plain water (H_2O) for 1 wk, were resolved by SDS-Polyacrylamide gel electrophoresis and subjected to western blotting by using an antibody against mouse SOD1. An anti-gapdh antibody was used as the loading control; B: Autoradiographs were scanned, and SOD1 expression was quantified by normalizing to gapdh expression. Normalized protein expression in water-fed *Sod2*$^{+/-}$ and alcohol-fed *Sod2*$^{+/-}$ and control mice was expressed as fold expression of that in water-fed control mice. LMC: Littermate control mice.

parts, alcohol-induced fold increases in total $O_2^{\bullet-}$ levels were higher in LMC mice than in *Sod2*$^{+/-}$ mice. However, 1 wk alcohol intake did not affect mitochondrial $O_2^{\bullet-}$ levels in both *Sod2*$^{+/-}$ and control mice, as shown by our MitoSOX Red and flow cytometry studies. Taken together, these findings suggest that short-term alcohol consumption elevates $O_2^{\bullet-}$ levels in cytoplasm and/or extracellular space, but not in mitochondria of hepatocytes. We can not however exclude the possibility of $O_2^{\bullet-}$ leaking out of mitochondria into the cytoplasm or alcohol-induced $O_2^{\bullet-}$ being rapidly converted to hydrogen peroxide, which is eliminated by glutathione peroxidase-1 in mitochondria and cytosol[15]. It is therefore possible that the livers of *Sod2*$^{+/-}$ mice are capable of preventing $O_2^{\bullet-}$ accumulation in mitochondria following short-term alcohol consumption.

Activated Kupffer cells (liver macrophages) are believed to be an important source of oxidants and play a pivotal role in the initial stages of ALD pathogenesis[28,49]. However, this study indicates that hepatocytes are also a significant contributor to the enhanced levels of free radical, particularly $O_2^{\bullet-}$, in the very early stages of alcohol consumption. Furthermore, our previously published studies indicate that Kupffer cells or the cytokine, tumor necrosis factor-α are not involved in the inhibition of hepcidin transcription by short-term alcohol exposure[50]. Kupffer cells have also been shown not to play a role in the regulation of hepcidin expression by iron or endotoxin *in vivo*[51].

Chronic alcohol consumption has been shown to elevate liver SOD2 expression but the effect of short-term alcohol intake is unclear[22]. We observed an increase in SOD2 protein expression in hepatocytes isolated from the livers of *Sod2*$^{+/-}$ mice following one week alcohol administration. However, this increase, which was absent in alcohol-treated LMC control mice, was not significant. Similarly, alcohol did not induce any significant changes in SOD2 mRNA levels in LMC control or *Sod2*$^{+/-}$ mice. Published studies report conflicting results regarding the role of SOD2 expression in ALD pathogenesis. Adenoviral delivery of SOD2 into rats has been shown to prevent liver injury following prolonged intragastric infusion of alcohol[35]. In contrast, another study reported that SOD2 over expression in mice worsens liver mitochondrial DNA

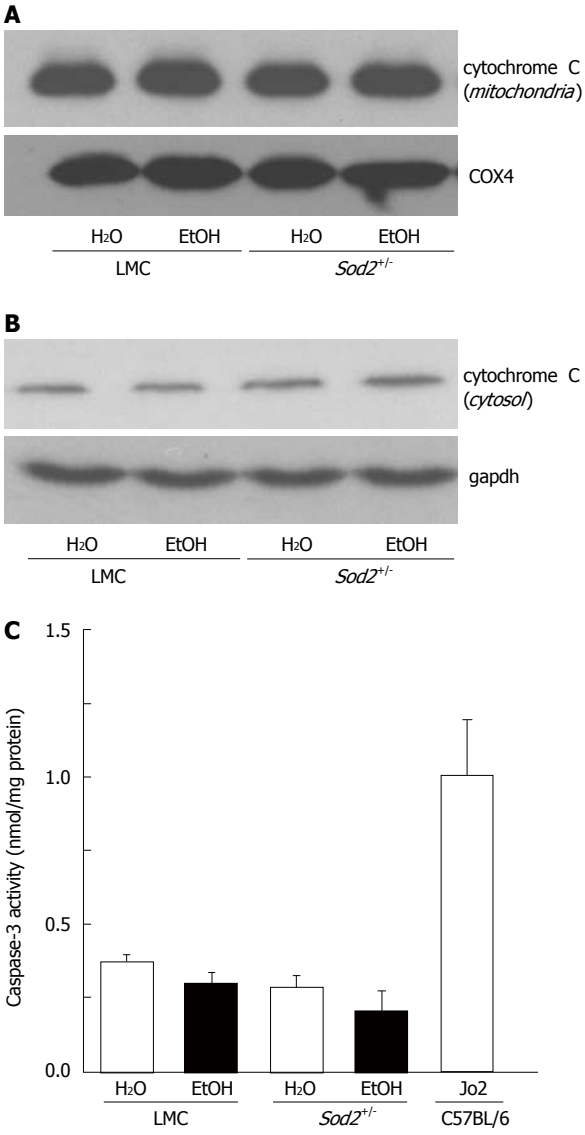

Figure 8 Detection of apoptosis in *Sod2*$^{+/-}$ mice with alcohol exposure. Liver protein lysates, isolated from mitochondria (A) or cytosol (B) of littermate control (LMC) and *Sod2*$^{+/-}$ mice fed with 10% ethanol (EtOH) or plain water (H_2O), were resolved by SDS-Polyacrylamide gel electrophoresis and subjected to western blotting by using an anti-cytochrome c antibody. Anti-COX4 (A) and gapdh (B) antibodies were used as loading controls; C: Whole cell lysate (WCL) proteins isolated from the livers of LMC and *Sod2*$^{+/-}$ mice fed with 10% ethanol or plain water were employed to measure caspase-3 activity, as described in Methods. WCL isolated from the livers of C57BL/6 strain wild-type male mice 6 h after the injection (*ip*) of anti-mouse Fas agonist antibody, Jo2 (0.32 μg/g. *b.w.*, Millipore) were used as internal control for caspase-3 assays.

depletion following prolonged alcohol consumption but prevents it after a single intragastric dose of alcohol exposure (*i.e.*, binge model)[36]. Accordingly, some dose-response studies have suggested that the addition or over expression of SOD can either be beneficial or detrimental by exacerbating cell injury or death (*i.e.*, hormesis)[16].

Of note, mitochondria are involved in the intrinsic pathway of apoptosis leading to the release of cytochrome C and caspase activation[52]. Van Remmen *et al*[53] reported apoptosis in cardiac tissues of *Sod2*$^{+/-}$ mice. Despite significant $O_2^{\bullet-}$ accumulation, the livers of untreated or short-term alcohol-fed *Sod2*$^{+/-}$ mice did not exhibit any caspase activation or mitochondrial cytochrome C release. Similarly, the livers of both global and liver-specific *Sod2* knockout mice have been shown not to exhibit any obvious morphological abnormalities[38,54]. Furthermore, studies by Cyr *et al*[55] using liver-specific *Sod2* knockout mice strongly suggest that hepatocytes have a reserve capacity to cope with the presence of mitochondrial ROS. Our findings therefore suggest that the existing defense mechanisms in the liver are sufficient to prevent both the accumulation of mitochondrial $O_2^{\bullet-}$ and apoptosis following short-term alcohol exposure.

Short-term alcohol exposure inhibited hepcidin transcription by three-fold in the livers of both *Sod2*$^{+/-}$ and LMC control mice. The fact that alcohol induced a similar level of hepcidin inhibition despite its differential effects on total $O_2^{\bullet-}$ production in LMC and *Sod2*$^{+/-}$ mice strongly suggests that $O_2^{\bullet-}$ is not involved in the regulation of hepcidin transcription by alcohol. Furthermore, the deletion of one allele of *Sod2* significantly elevated both total and mitochondrial $O_2^{\bullet-}$ levels but did not alter basal hepcidin expression in the livers of *Sod2*$^{+/-}$ mice compared to LMC mice. Hepcidin expression has been shown to be regulated by H_2O_2 in tissue culture cell models[56]. We can not exclude a role for other free radicals in the regulation of hepcidin and thereby iron metabolism in ALD, which will be investigated in future studies.

In a conclusion, Patients with ALD and animal models of ALD frequently exhibit iron overload. Hepcidin, which is primarily synthesized in the hepatocytes of the liver, is the pivotal regulator of iron homeostasis. We have previously demonstrated that the inhibition of liver hepcidin expression (and the accompanying increase in intestinal iron absorption) by short-term alcohol exposure can be prevented by antioxidants, vitamin E and N-acetylcysteine *in vivo*. This study investigates the role of mitochondrial $O_2^{\bullet-}$ in this process by using knockout mice heterozygous for *Sod2* (*Sod2*$^{+/-}$) and LMC mice. EPR analysis indicated that 1 wk of moderate alcohol consumption is sufficient to significantly elevate $O_2^{\bullet-}$ levels in hepatocytes freshly isolated from the livers of untreated and alcohol-treated *Sod2*$^{+/-}$ and LMC mice. This alcohol-mediated increase in $O_2^{\bullet-}$ did not however originate from mitochondria, as shown by MitoSOX and flow cytometry studies. Despite the significant differences in hepatic $O_2^{\bullet-}$ levels, *Sod2*$^{+/-}$ and LMC mice displayed a similar level of hepcidin inhibition following alcohol administration. $O_2^{\bullet-}$ accumulation in untreated *Sod2*$^{+/-}$ mice did not alter the basal expression level of hepcidin in the liver. Collectively, our studies strongly suggest that mitochondrial superoxide is not involved in the inhibition of liver hepcidin expression and thereby regulation of iron metabolism by alcohol. Further, our data indicate that hepatocytes are a significant source of oxidants in the very early stages of ALD progression.

COMMENTS

Background

Patients with alcoholic liver disease (ALD) and animal models of ALD frequently

exhibit iron overload. However, the underlying mechanisms are not well understood. The authors' laboratory and others have shown that both short-term and long-term alcohol intake inhibits the expression of hepcidin in the liver *in vivo*.

Research frontiers

Iron and alcohol act synergistically to induce liver injury. Hepcidin, which is primarily synthesized in the hepatocytes of the liver, is the pivotal regulator of iron homeostasis. Alcohol-induced oxidative stress in the liver is involved in the pathogenesis of ALD. It is therefore important to understand the regulation of hepcidin by alcohol-induced oxidative stress.

Innovations and breakthroughs

The authors have previously demonstrated that short-term and moderate alcohol consumption is sufficient to inhibit liver hepcidin expression leading to an increase in intestinal iron transporter expression *in vivo*. Interestingly, both of these processes were abolished by antioxidants, vitamin E and N-acetylcysteine, strongly suggesting a role for alcohol-induced oxidative stress. Several elegant studies have shown the importance of oxidative stress and mitochondria in alcohol-induced liver injury but the individual role of free radicals is unclear. This study investigates the role of alcohol-induced superoxide generation in the regulation of hepcidin in the liver.

Applications

The findings in this study suggest that hepatocytes are a significant source of oxidants in the very early stages of ALD progression. Moderate and short-term alcohol consumption is sufficient to significantly elevate hepatic superoxide levels, which do not however originate from mitochondria. Further, alcohol-induced superoxide is not involved in the inhibition of liver hepcidin expression. These findings will help us to further understand the mechanisms of liver injury with the ultimate aim of developing novel diagnostic and treatment strategies for ALD.

Terminology

Liver is important for both alcohol and iron metabolism. The generation of free radicals and lipid peroxidation plays a major role in the pathogenesis of ALD. The free radical, superoxide ($O_2^{\cdot-}$) can initiate lipid peroxidation, and iron can interact with $O_2^{\cdot-}$ and hydrogen peroxide to yield hydroxyl radical, which is highly reactive and detrimental. The antioxidant enzyme, superoxide dismutase (SOD) plays a protective role by catalyzing the dismutation of $O_2^{\cdot-}$ into hydrogen peroxide and oxygen.

Peer review

This work utilizes *Sod2*$^{+/-}$ mice to address the role of SOD2 and superoxide in alcohol-mediated regulation of hepcidin and iron metabolism. The authors are pioneers in this field.

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P- Reviewers: Pantopoulos K, Xu J **S- Editor**: Wen LL
L- Editor: A **E- Editor**: Yan JL

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.4331/wjbc.v4.i4.131

World J Biol Chem 2013 November 26; 4(4): 131-140
ISSN 1949-8454 (online)

ORIGINAL ARTICLE

High-density lipoprotein endocytosis in endothelial cells

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Supported by the Austrian Science Fund, No. P20116-B13 and No. P22838-B13
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Received: August 30, 2013 Revised: November 11, 2013
Accepted: November 18, 2013
Published online: November 26, 2013

Abstract

AIM: To describe the way stations of high-density lipoprotein (HDL) uptake and its lipid exchange in endothelial cells *in vitro* and *in vivo*.

METHODS: A combination of fluorescence microscopy using novel fluorescent cholesterol surrogates and electron microscopy was used to analyze HDL endocytosis in great detail in primary human endothelial cells. Further, HDL uptake was quantified using radio-labeled HDL particles. To validate the *in vitro* findings mice were injected with fluorescently labeled HDL and particle uptake in the liver was analyzed using fluorescence microscopy.

RESULTS: HDL uptake occurred *via* clathrin-coated pits, tubular endosomes and multivesicular bodies in human umbilical vein endothelial cells. During uptake and resecretion, HDL-derived cholesterol was exchanged at a faster rate than cholesteryl oleate, resembling the HDL particle pathway seen in hepatic cells. In addition, lysosomes were not involved in this process and thus HDL degradation was not detectable. *In vivo*, we found HDL mainly localized in mouse hepatic endothelial cells. HDL was not detected in parenchymal liver cells, indicating that lipid transfer from HDL to hepatocytes occurs primarily *via* scavenger receptor, class B, type I mediated selective uptake without concomitant HDL endocytosis.

CONCLUSION: HDL endocytosis occurs *via* clathrin-coated pits, tubular endosomes and multivesicular bodies in human endothelial cells. Mouse endothelial cells showed a similar HDL uptake pattern *in vivo* indicating that the endothelium is one major site of HDL endocytosis and transcytosis.

Key words: High-density lipoprotein; Endocytosis; Endothelium; Human umbilical vein endothelial cells; Human coronary artery endothelial cells; Cholesterol

Core tip: The cardio-protective effect of high-density lipoprotein (HDL) is related to its ability to transfer lipids from the periphery, such as atherosclerotic plaques, back to the liver for excretion. Therefore, HDL has to cross the endothelial barrier. In the present work we analyzed the steps and way stations of HDL uptake and resecretion using novel fluorescent cholesterol surrogates in human endothelial cells as a model for the endothelial barrier. HDL uptake occurred *via* clathrin-coated pits, tubular endosomes and multivesicular bod-

ies in human umbilical vein endothelial cells. Finally we compared key findings to the *in vivo* situation.

Fruhwürth S, Pavelka M, Bittman R, Kovacs WJ, Walter KM, Röhrl C, Stangl H. High-density lipoprotein endocytosis in endothelial cells. *World J Biol Chem* 2013; 4(4): 131-140 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i4/131.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i4.131

INTRODUCTION

Plasma concentrations of high-density lipoprotein (HDL) cholesterol exhibit an inverse association with the incidence of cardiovascular diseases. The cardio-protective effect of HDL is related to its ability to transfer lipids from the periphery back to the liver for excretion into the bile. This cholesterol clearance is called reverse cholesterol transport[1]. To achieve the removal of excess cholesterol deposited in the arterial intima, HDL must first cross the endothelial barrier to get into close proximity to macrophage foam cells found in atherosclerotic plaques. The mechanisms and way stations in this uptake and resecretion process of HDL seem to be redundant as several receptors mediate HDL uptake. Thus its details and the interplay of these receptors in the transport of HDL and its derived lipids across cells are not fully understood (for review see[2]).

Endocytosis and resecretion of HDL was first described by Bierman *et al*[3] and Stein *et al*[4] in rat aortic smooth muscle cells (for review see[5]). Bierman *et al*[3] suggested regurgitation of non-catabolized apolipoproteins by reverse endocytosis of HDL. Schmitz *et al*[6] described the interaction of HDL with cholesteryl ester-laden macrophages; subsequent to receptor-mediated binding, HDL internalization and transport into endosomes were demonstrated. These macrophages did not degrade HDL but rather resecreted internalized HDL particles on a path similar to the transferrin receptor[6]. Retroendocytosis of HDL particles was also demonstrated in a rat liver cell line[7]. During internalization, HDL is remodeled to larger apoE-containing HDL_2-like particles[8]. Endocytosis and resecretion is not limited to HDL as it occurs for almost all lipoprotein classes: uptake and resecretion was described also for low density lipoprotein (LDL) or very LDL (VLDL)[3,9-13]. Additionally, apolipoprotein E (apoE) recycling has been reported to occur in hepatocytes and macrophages, where a part of the apoE associated with HDL escapes degradation[14-16] (for review see[17]).

In general, transport of molecules across barriers is determined by the water solubility, the size and charge of the corresponding molecule. Lipoproteins as well as apolipoprotein A- I (apoA- I) have been shown to be endocytosed/transcytosed in polarized hepatocytes and epithelial cells including endothelial cells[2,7,18-30]. Besides transendothelial transport, proteins can overcome the endothelial barrier by paracellular transport. The latter involves the modulation of interendothelial junctions in order to transport molecules larger than 6 nm (for review see[2]).

Scavenger receptor, class B, type I (SR-BI) has been shown to be involved in HDL particle uptake in polarized hepatocytes[22]. Ablation of SR-BI is associated with deregulation of cholesterol homeostasis in the arterial wall, thereby increasing the susceptibility to atherosclerosis[31]. Besides SR-BI, ATP binding cassette transporter A1 (ABCA1), ATP binding cassette transporter G1 (ABCG1), caveolin-1 and ecto-F_1-ATPase are considered to be involved in HDL/apoA-I uptake or transcytosis[2,23,24,32-37]. Recently, transport of HDL back to the liver was demonstrated to occur *via* lymphatic vessels, with SR-BI being the main receptor mediating transcytosis of HDL across the lymphatic endothelium[38,39].

In this project we analyzed HDL uptake in endothelial cells. Therefore, we used light and electron microscopical methods enabling the visualization of HDL particles *via* crosslinking and their derived lipids using novel fluorescent cholesterol surrogates. Overall the process of HDL transfer must encompass: (1) binding of HDL to the apical side of the endothelial cells to receptors/proteins and its concomitant uptake; (2) transport of HDL particles and their cholesterol/cholesteryl esters to the basolateral side of the endothelial cells; and (3) excretion of HDL at the basolateral side of the endothelial cells. We demonstrate that HDL uptake *via* clathrin-coated pits leads to a rapid exchange of the cholesterol backbone visualized *via* a novel cholesterol surrogate. Furthermore, HDL was transported to multivesicular bodies without concomitant degradation, indicative of HDL resecretion.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) (PromoCell, Germany) were cultured in flasks coated with 0.5% gelatin in Endothelial Cell Growth Medium (PromoCell) containing endothelial cell growth supplement, epidermal growth factor, basic fibroblast growth factor, heparin and hydrocortisone, supplemented with 5% fetal calf serum (PromoCell). Passages from 4 to 10 were used for the experiments. Prior to experiments, the medium was changed to serum-free Endothelial Cell Growth Medium containing the endothelial cell growth supplement mix (PromoCell).

Animal treatment

Animal experiments were conducted at the Swiss Federal Institute of Technology Zürich. All protocols for animal use and experiments were reviewed and approved by the Veterinary Office of Zurich (Switzerland). Male C57BL/6 mice (8 wk; 18-20 g) were obtained from the Jackson Laboratory (Bar Harbor, United States). Animals were kept on chow under standard conditions. For

experiments, 200 μg of HDL-Alexa-568 (resembling 1/10 of total murine HDL) were injected into the tail vein. Mice had ad libitum access to water and were restrained from food afterwards. Mice were anesthetized by intraperitonal injection of Ketamin (40 mg/kg BW; Vetoquinol, Ittigen, Switzerland) and Rompun (2 mg/kg BW; Bayer HealthCare, Germany) after 1 h. Transcardial perfusion was performed with a 30 g needle at a rate of 3.2 mL/min with saline (1 min) and 4% formaldehyde/0.5% glutaraldeyde in PBS (14 min). Organs were collected and postfixed in 4% formaldehyde in PBS overnight.

Lipoprotein isolation and labeling with fluorescent dyes

HDL was prepared from plasma of healthy volunteers by sequential ultracentrifugation (d = 1.21 g/mL)[40]. The apolipoprotein part of HDL was covalently labeled with an Alexa Fluor 568 dye (Molecular Probes, United States) according to the manufacturer's instructions. Additional loading of HDL with Bodipy-cholesteryl oleate (BP-CE) or Bodipy-cholesterol (BP-C) was done as described previously[41]. HUVECs and HCAECs were incubated with 50 μg/mL labeled HDL for up to 60 min. Cells were subsequently fixed in 4% para-formaldehyde at 4 ℃ for 30 min, washed twice with PBS, mounted with Fluoprep (Biomerieux, France) and imaged using a confocal microscope (LSM 5 Exciter, Zeiss, Germany). Alternatively, cells were incubated with labeled HDL for 60 min and subsequently lyzed using 1% cholate. Fluorescence intensities of Alexa 568 and the Bodipy label were measured using a fluorometer (Zenyth 3100, Anthos, Germany).

[^{3}H-CE-, ^{125}I-]-HDL labeling and uptake experiments

HDL was labeled with sodium [^{125}] iodine (Hartmann Analytics, Germany) alone or additionally with [^{3}H]-cholesteryl-oleate (Perkin Elmer, United States) as described previously[42]. For uptake experiments HUVECs were seeded in 12-well plates. To calculate unspecific binding, a 40-fold excess of unlabeled HDL was added to every fourth well. [^{3}H-CE-, ^{125}I]-HDL or [^{125}I]-HDL was added to each well at a final concentration of 10 μg/mL. After up to 6 h, cell supernatants were collected for degradation measurements, which were performed as previously described[43]. Cells were washed twice with cold PBS + BSA (2 mg/mL) and twice with cold PBS. Cells were then lysed with 0.1 mol/L NaOH. [^{125}I-] radioactivity in the lysates was counted using a gamma-counter (COBRAII Auto-gamma; Perkin Elmer). [^{3}H]-radioactivity was counted using 15 mL Ready-Safe (Beckman Coulter; United States) and a beta-counter (Tri-Carb 2800TR; Perkin Elmer). Measurements were normalized to protein content, determined by the Bradford protein assay (Biorad, Germany).

Immunofluorescence microscopy on HUVECs

After incubation with HDL-Alexa 568 for 1 h, HUVECs were fixed in 4% para-formaldehyde at 4 ℃ for 30 min. For immunofluorescence staining, cells were permeabilized with ice-cold methanol for 20 s and blocked with 2.5% BSA in PBS for 30 min. Afterwards, samples were incubated with the primary antibody to LIMP Ⅱ (Novus Biologicals, United States) diluted 1:200 in 1% BSA in PBS (buffer A) containing 1% horse serum for 1 h. Samples were then rinsed 3 times with buffer A containing 0.1% TWEEN-20. Next, cells were incubated with the secondary Alexa 488 conjugated antibody (Molecular Probes) diluted 1:250 in buffer A containing 1% horse serum. Finally cells were rinsed 3 times with buffer A containing 0.2% TWEEN-20, once with PBS and mounted with Fluoprep (Biomerieux). Cells were imaged using a fluorescence microscope (Axiovert 135, Zeiss).

Immunofluorescence microscopy on liver sections

Immunofluorescence was performed on free-floating 50 μm vibratome sections, because paraffin embedding quenched the Alexa signal. Sections were blocked with 4% BSA in PBS + 0.05% Tween-20 for 2 h and incubated with the following primary antibodies in PBS + 1% BSA + 0.05% Tween-20 over-night: anti-CD34 (Abcam ab8536; 1/100), anti-CD14 (Sigma HPA001887; 1/200) and anti-desmin (Sigma D1033; 1/50). After 5 washing steps with PBS, sections were incubated with secondary antibodies coupled to Alexa-488 (in PBS + 1% BSA + 0.05% Tween-20) for 2 h. After another 5 washing steps with PBS, samples were counterstained with TO-PRO-3 (Invitrogen), mounted and analyzed using a LSM 510 laser scanning confocal microscope (Zeiss, Germany).

Electron microscopy

Horseradish peroxidase (HRP) labeled HDL was prepared using the peroxidase labeling kit (Roche, Switzerland) according to the manufacturer's instructions. HUVECs were incubated with 25 μg/mL HDL-HRP for 30 min. Cells were fixed in 0.1 mol/L cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde at 4 ℃ for 45 min. Cells were then washed twice with cacodylate buffer and twice with 0.05 mol/L Tris-HCl buffer (pH 7.6). HDL-HRP was visualized using the classical DAB-oxidation reaction[44]. Cells were postfixed in OsO_4-ferrocyanide (15 min) and in 1% OsO_4-veronalacetate (2 h). Samples were further processed for electron microscopy; after dehydration in a gradient of aqueous ethanol (70% overnight at 4 ℃; 80%, 96% and 100% for 10 min at room temperature each). Afterwards they were embedded in Epon (Serva, Germany), 80-100 nm sections were cut with an UltraCut-UCT ultramicrotome (Leica Inc., Austria). Samples were transferred to copper grids, post-stained with uranyl acetate and lead citrate and examined with a Zeiss EM900 transmission electron microscope equipped with a wide-angle Dual speed CCD camera (Albert Tröndle Dünzelbach, Germany).

Statistical analysis

Results were expressend as mean ± SD. Data were analyzed using a two-tailed Student's *t*-test.

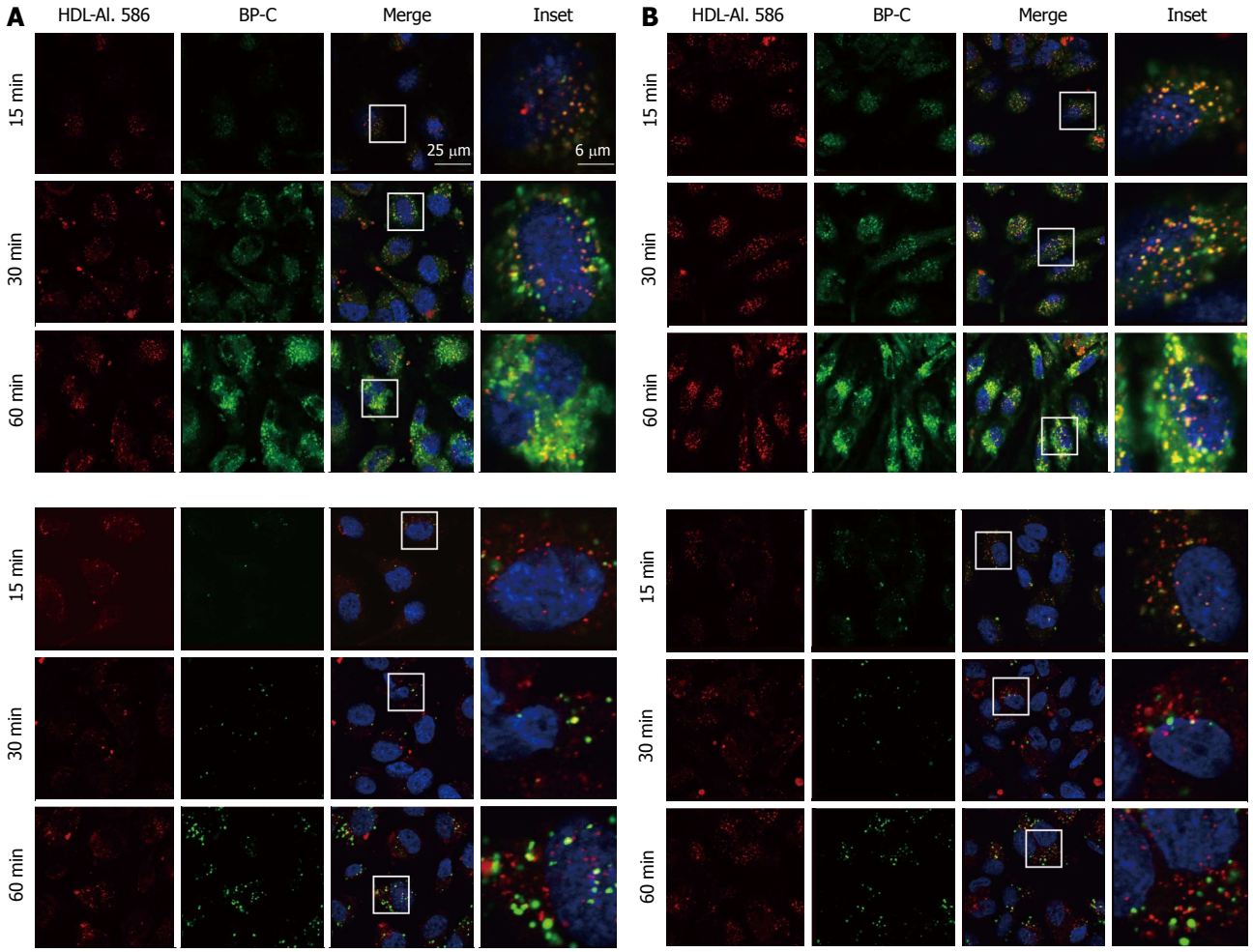

Figure 1 Detailed analysis of high density lipoprotein uptake and high density lipoprotein -derived lipid transfer in human umbilical vein endothelial cells and human coronary artery endothelial cells. A: HUVECs; B: HCAECs. HUVECs and HCAECs were incubated for the indicated time with HDL-Alexa 586, which was additionally labeled with either Bodipy-cholesterol (BP-C; upper panel) or Bodipy-cholesteryl oleate (BP-CE; lower panel). Samples were fixed and imaged using confocal microscopy. Note that brightness and contrast were increased at the 15 min time point of the BP-CE panel for better visibility (lower panel). HUVECs: Human umbilical vein endothelial cells; HCAECs: Human coronary artery endothelial cells; HDL: High density lipoprotein.

RESULTS

Uptake of HDL and its derived lipids in human endothelial cells

HDL uptake and transcytosis occur in endothelial cells[23] but the uptake path of HDL and the exchange behavior of its derived lipids during this process is elusive. To visualize this lipid transfer we directly labeled the apolipoprotein part of HDL covalently with Alexa 568 and the lipid part with a novel cholesterol surrogate. As HDL transports cholesterol in both its free and esterified form, we utilized Bodipy-cholesterol (BP-C) as well as Bodipy-cholesteryl oleate (BP-CE) as cholesterol surrogate markers. Size and shape of the reconstituted HDL particles containing either BP-C or BP-CE was assessed previously[41]. They were shown to be discoidal particles with a size comparable to native HDL. Both double-labeled HDL particles were applied to human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) for the indicated time (Figure 1). HDL particle uptake occurred already after 5 min of incubation (not shown) and further proceeded and increased over a 3 h incubation period in HUVECs. HDL-Alexa 568 exhibited a vesicular transport pattern with some enrichment in the perinuclear area. Next, we determined the fate of both the apolipoprotein part and the lipid part of the HDL particle during endocytosis. Within 15 min both HDL-Alexa 568 and BP-C were clearly detected intracellularly in both HUVECs and HCAECs (Figure 1 upper panels). At this time point both labels still co-localized. With increasing time (up to 60 min) this co-localization decreased and BP-C accumulated mainly in the perinuclear area, indicating that cholesterol underwent transfer from the HDL particle to the cell. When BP-CE was employed as a marker for esterified cholesterol, the uptake of both the lipid and protein part occurred in parallel (Figure 1 lower panels), indicating that most of the esterified cholesterol during the 30 min uptake period is still contained within the HDL particle. After 60 min some of the BP-CE has been transferred to the perinuclear area. These data indicate that the transfer/exchange of free cholesterol between the HDL particle and the cells occurs faster than the transfer of esterified

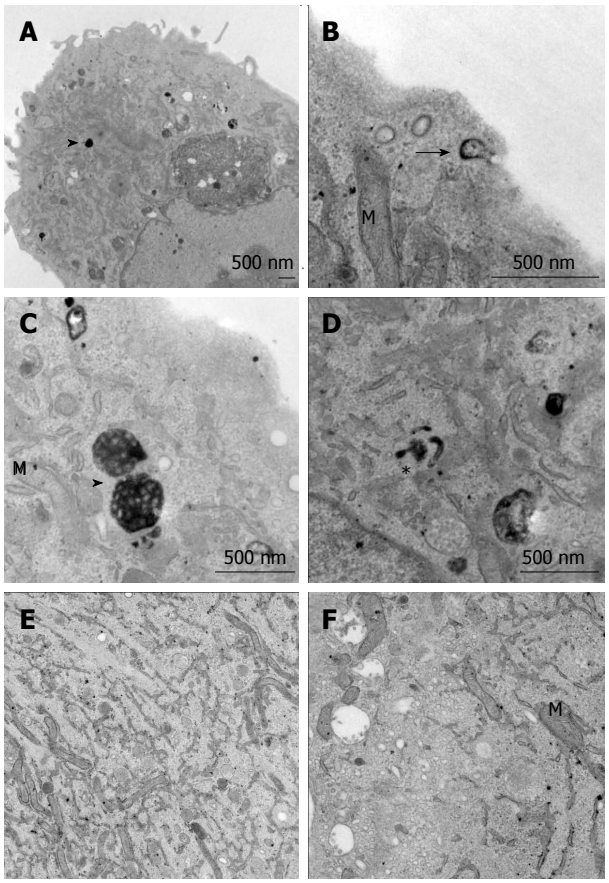

Figure 2 Ultrastructural analysis of high density lipoprotein uptake in human umbilical vein endothelial cells. Cells incubated with HRP-labeled HDL for 30 min (A-D) or untreated controls (E, F) were processed for electron microscopy. HRP-positive clathrin-coated vesicles were seen near the cell surface (arrow in B), in tubular endosomes (asterisk in D), and densely packed MVBs were found in the perinuclear area (arrow heads in A and C). No specific staining was seen in control samples (E, F). HRP: Horseradish peroxidase; HDL: High density lipoprotein; M: Mitochondria.

cholesterol.

To analyze the way stations of HDL endocytosis we labeled the HDL particle covalently in the protein moiety with HRP, which can be visualized by transmission electron microscopy. HRP-HDL was clearly detected at the cell surface in clathrin-coated vesicles in HUVECs after 30 min of incubation. Furthermore, HDL was endocytosed and seen in tubular endosomes and multivesicular bodies (Figure 2). This HDL uptake pattern is similar to the one described earlier by our group in hepatic cells[41].

HDL particles exchange lipids during the transfer process

To quantify HDL uptake in HUVECs the protein part of the HDL particle was radioactively labeled with iodine and the lipid moiety was loaded with [^{3}H]-cholesteryl oleate. HUVECs were incubated for 1 h with the double-labeled HDL particles and specific HDL cell association was analyzed. Unspecific [^{3}H-CE-, ^{125}I-]-HDL cell association was measured in the presence of a 40-fold excess of unlabeled HDL, which competed for labeled HDL cell association. Within this hour HDL cell association was 128 ± 51 ng HDL/mg cell protein (n = 2). A time course study of HDL cell association (0-6 h) showed the expected saturation curve (Figure 3A). HDL degradation was below the detection limit (not shown). Furthermore, Alexa-HDL did not co-localize with the lysosomal marker LIMPII in HUVECs (Figure 3B) indicating again that the uptake route of HDL does not involve lysosomes. Next, [^{3}H]-cholesteryl oleate transfer was quantified. During a time period of 1 h an equivalent of 3787 ± 918 ng HDL/mg cell protein for [^{3}H]-cholesteryl oleate, showing that lipid transfer was detected (n = 3). To follow cholesterol and cholesteryl oleate delivery to HUVECs in detail, we again used the fluorescent cholesterol surrogates. After HDL uptake for 1 h the ratio of the apolipoprotein label Alexa 568 to BP-C changed (Figure 3C); after labeling HDL particles had an Alexa 568/BP-C ratio of 2.6, and after 1 h this ratio inverted to 0.9 in the cell lysate, indicating transfer of BP-C from the HDL particle to the cells. A similar behavior was observed for BP-CE. The initial ratio of Alexa 568/BP-CE was 7.1, and this ratio was altered even to a higher extent to 1.2, again providing evidence for remodeling of the HDL particles during the endocytosis process. We found no uptake of HDL-Alexa 568 at 4 ℃, indicating that holo-HDL particle uptake was inhibited. BP-C uptake from the double labeled particles was largely decreased at 4 ℃ and BP-CE uptake was completely blocked, indicating that lipid transfer from HDL was also temperature dependent (data not shown).

HDL uptake in mouse endothelium

To confirm the *in vitro* data, we administered HDL-Alexa 568 *via* tail vein injection into C57BL/6 mice. Mice were sacrificed after 1 h, and liver sections were processed for immunohistochemistry. We used CD34 as marker for endothelial cells. Figure 4 shows co-localization of Alexa 568, the marker for the HDL particle, and CD34. Both CD34 and HDL-Alexa 568 showed intense staining in cells lining the sinusoid vessels. In addition, liver sections were also stained with CD14 and desmin, which are markers for Kupffer and stellate cells, respectively (Figure 4). Almost no co-localization of HDL Alexa 568 with CD14 or desmin was found, indicating that these cells did not bind and take up HDL to a significant amount within 1 h. The intensity of HDL staining did not differ between periportal and pericentral regions. HDL staining was predominantly found in sinusoidal endothelial cells and not in the endothelial cells of larger vessels (*i.e.*, central vein and portal vein). Taken together, these data demonstrate that the Alexa-labeled HDL particles can be used to follow HDL uptake *in vivo* and that transfer routes and possibly lipid exchange can be monitored in the *in vivo* setting.

DISCUSSION

Transendothelial transport of proteins occurs *via* para- or

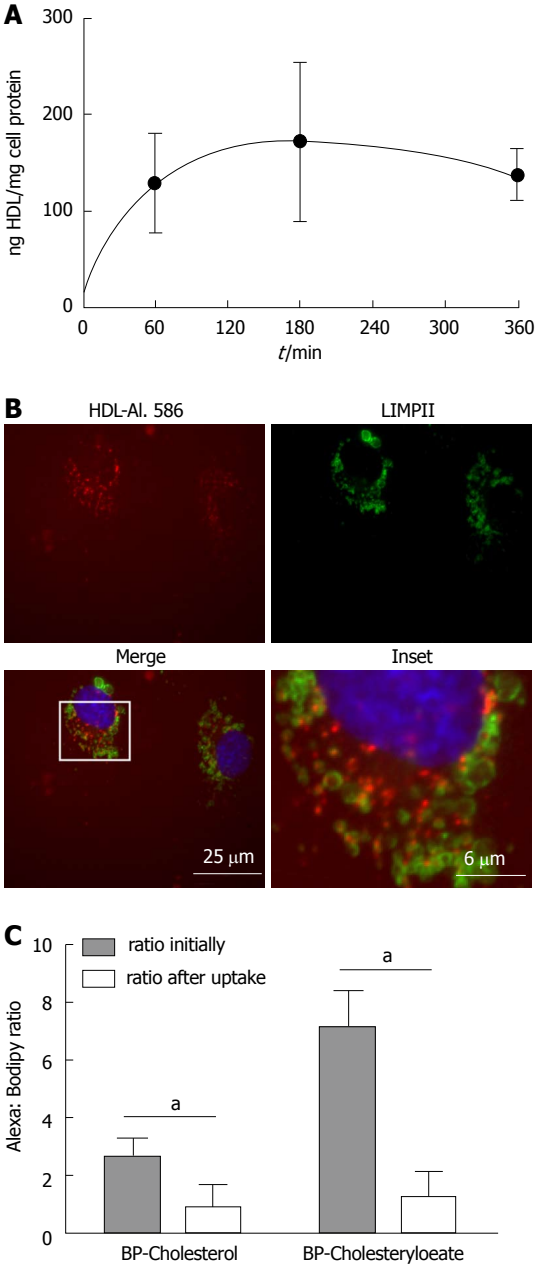

Figure 3 Quantification of high density lipoprotein uptake. A: Time course of HDL uptake; HUVECs were incubated with iodinated HDL for up to 6 h and specific cell association was measured using a gamma counter. Measurements were normalized to protein content, determined by the Bradford protein assay; B: HUVECs were incubated with Alexa 568 labeled HDL for 1 h. Cells were fixed and immunofluorescene was performed using an antibody against LIMPII and visualized using fluorescence microscopy. No colocalization of LIMPII, a marker for the lysosomal compartment, and HDL-Alexa 568 was seen; C: HUVECs were incubated with HDL-Alexa-BP-C or -BP-CE for 60 min. Cells were then lyzed and the fluorescence intensity for each label was measured using a fluorometer. Results are expressed as the Alexa: Bodipy ratio initially and after the 60 min incubation period (n = 3). $^{a}P < 0.05$ between groups. HDL: High density lipoprotein; BP-C: Bodipy-cholesterol; BP-CE: Bodipy-cholesteryl oleate; HUVECs: Human umbilical vein endothelial cells.

trans-cellular pathways, with transport of HDL being described to proceed *via* transcytosis[23,37-39]. For this, HDL must be endocytosed in the first step, and then the particle is transported intracellularly from the apical to the basolateral side of the cell, and finally the particle itself is excreted. In the present work we performed a detailed morphological analysis of HDL endocytosis and the transfer of its lipids using novel cholesterol surrogates in endothelial cells. HDL uptake occurred *via* clathrin-coated pits, leading to a rapid exchange of cholesterol cargo (Figures 1 and 3). Intracellularly, HDL was transported *via* endosomes to multivesicular bodies (Figure 2) to await its resecretion. This endocytosis path was similar to earlier observations in hepatic cell lines[41]. *In vivo* mouse endothelial cells in the liver exhibited a comparable HDL uptake pattern.

Here we report that HDL was detected at the cell surface in clathrin-coated invaginations using DAB staining and electron microscopy (Figure 2), indicative clathrin-mediated endocytosis. Indeed, using HepG2 cells, we saw a similar binding and uptake behavior of HDL, with the involvement of endosomal vesicles and multivesicular bodies in the uptake process[41]. This uptake path is similar to that reported here for endothelial cells. In addition, in both cases lysosomes were not involved in this process and thus HDL degradation was not detected. HDL catabolism is limited to certain tissues such as the kidney (for review see[45]). Hepatic HDL catabolism is disturbed in cases of imbalanced metabolism like obesity, with leptin being one regulator, as in *ob/ob* mice HDL degradation in the liver is decreased[46]. We did not find an involvement of caveolae in this uptake process; however, SR-BI, which seems to participate in endothelial HDL uptake at least in part, was reported to co-precipitate with caveolin[47]. As the caveolar density differs considerably between different cell types, the involvement of caveolae in HDL endocytosis might be cell type specific. For instance, we found strong HDL staining in caveolae in CHO cells using HDL-HRP staining (data not shown). Taken together, the data indicate that HDL and its cargo are taken up by endothelial cells *via* a vesicular path involving clathrin-coated vesicles and multivesicular bodies, with HDL catabolism being low.

In general, HDL metabolism has to be very versatile in order to adapt to the different environments such as peripheral tissues or the central cholesterol distributor, the liver. Thus HDL/lipid transport is complex, with the existing cholesterol gradient being one driving force at least for free cholesterol exchange. In addition, selective cholesteryl ester uptake from HDL particles occurs mainly in liver and adrenal, with HDL degradation being low in these tissues[48]. During HDL endocytosis, the lipid moiety of the HDL particle is altered massively. Using the novel fluorescent cholesterol surrogates Bodipy-cholesterol and Bodipy-cholesteryl oleate (Figure 1) or double-radiolabeled HDL particles cholesterol and cholesteryl ester, which were contained within the HDL particle before endocytosis, were localized intracellularly after uptake. These data are in agreement with our previous work on HepG2 cells[41]. Some studies

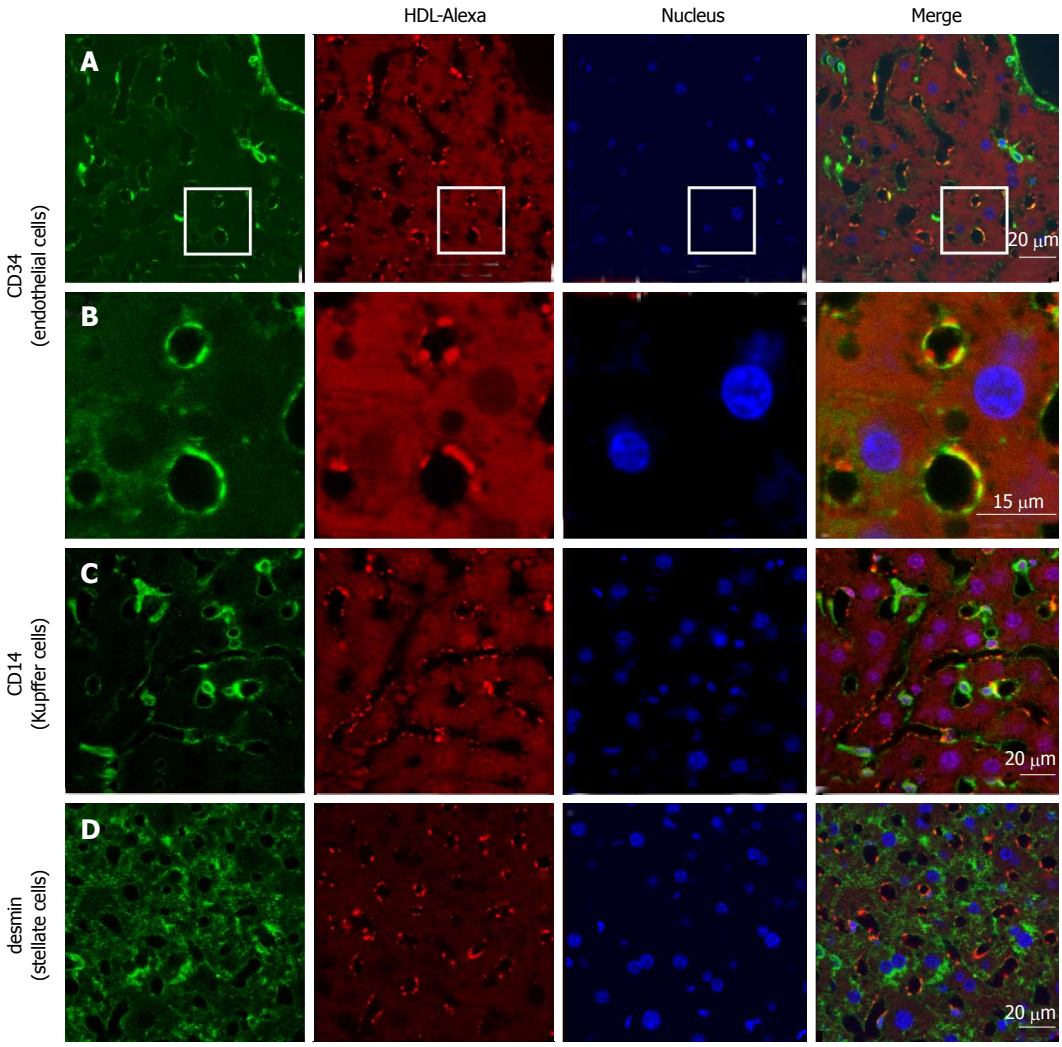

Figure 4 High density lipoprotein uptake in non-parenchymal liver cells. Fluorescently labeled high density lipoprotein (HDL)-Alexa 568 was intravenously injected into C57BL/6 mice. After 60 min, tissues were fixed by transcardial perfusion. Liver sections were stained for cellular markers by immunofluorescence and analyzed by confocal microscopy. HDL is localized in endothelial cells [A and B (inset)] and to a limited amount in Kupffer cells (C), whereas stellate cells show no detectable HDL staining (D).

have reported different transport routes for HDL and its associated free sterol in polarized hepatocytes[20,22,30], with the endoplasmic recycling compartment and multivesicular bodies being the main transfer sites of HDL cargo.

Until now, HDL uptake was visualized in *in vitro* systems, but in the present work we extended these studies to mice. Alexa-labeled HDL was delivered to mice by tail-vein injection and after 1 h the label was detected in endothelial cells of the liver. Out *et al*[49] reported that SR-BI was not involved in the transfer of HDL-derived lipids to endothelial cells of mouse liver, as SR-BI ablation did not lead to an alteration in this uptake. This suggests an involvement of clathrin-coated pits rather than caveolae in the uptake process. In mice, we observed strong staining of HDL-Alexa 568 in endothelial cells but not in Kupffer or stellate cells (Figure 4). As there are still some discrepancies between *in vitro* and *in vivo* data, more work is needed to assess HDL endocytosis in animal models. A recent study showed that HDL transcytosis occurs with the involvement of lymphatic vessels in the removal of cholesterol from peripheral tissues on its way back to the liver for disposal[39].

In summary, we visualized HDL uptake and its lipid exchange in endothelial cells *in vitro* and *in vivo*. The endothelium is one major site of HDL endocytosis and transcytosis, while other liver cell types seem to play a minor role in HDL endocytosis.

ACKNOWLEDGMENTS

We thank Jelena Brankovic and Regina Wegscheider for excellent technical assistance.

COMMENTS

Background

High-density lipoprotein (HDL) particles transfer lipids from the periphery to the

liver for excretion into the bile. This so-called reverse cholesterol transport is one mechanism thought to contribute to the anti-atherosclerotic effects of HDL. To remove cholesterol from macrophages residing in the arterial intima and thus preventing atherosclerotic plaque progression, HDL has to cross the endothelial barrier. However, the route by which HDL crosses the endothelial barrier and many details concerning its travel back to the liver *via* lymphatic vessels are still enigmatic.

Research frontiers

In order to excert its main anti-atherosclerotic function, HDL has to cross the vascular endothelium to reach atherosclerotic lesions. This transport can either involve transcytosis of the particles with endocytosis in a first step or paracellular transport of HDL. The authors show that HDL endocytosis occurs *via* clathrin-coated pits, tubular endosomes and multivesicular bodies in primary human endothelial cells. In the present study, the authors provide evidence for the important role of HDL endocytosis in endothelial cells also *in vivo*.

Innovations and breakthroughs

In this project, the authors characterized HDL endocytosis in endothelial cells *in vitro* and *in vivo* in great detail. Therefore, the authors used light and electron microscopical methods enabling the visualization of HDL particles and their derived lipids using novel fluorescent cholesterol surrogates. Moreover the authors injected fluorescently labeled HDL into mice and analyzed HDL uptake in hepatocytes *in vivo*. Taken together the authors found that endothelial cells play an important role in HDL endocytosis *in vitro* and *in vivo*.

Applications

The role of endothelial HDL endocytosis and transcytosis during atherogenesis and in atherosclerotic lesions is not clear. Our study indicates that the endothelium is a major site of HDL endocytosis and therefore there is need for future studies.

Terminology

Atherosclerosis is a thickening of the arteries, a stepwise process which starts with the accumulation of excess lipids in the arterial wall. The so called atherosclerotic plaque can subsequently rupture and block arteries leading for example to stroke. Atherogenesis is the development of atherosclerosis and influenced by many factors, such as lipid and lipoprotein levels.

Peer review

This is a convincing study elucidating the distribution of cholesterol and cholesteryl esters during HDL uptake. The combination of new fluorescent marker techniques, classical combined radio-labeling approaches, electron microscopy and fluorescence microscopy is a strength of this carefully designed study.

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P- Reviewers: Cairo G, Cheng XD, Gotte M, Liu T
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Yan JL

World Journal of Biological Chemistry

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.4331/wjbc.v4.i4.141

World J Biol Chem 2013 November 26; 4(4): 141-147
ISSN 1949-8454 (online)

BRIEF ARTICLE

Changes in aminoacidergic and monoaminergic neurotransmission in the hippocampus and amygdala of rats after ayahuasca ingestion

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Supported by Fundação de Amparo a Pesquisa do Estado de São Paulo; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; Conselho Nacional de Desenvolvimento Científico e Tecnológico; Instituto Nacional de Neurociência Translacional
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Received: May 12, 2013 Revised: September 29, 2013
Accepted: October 17, 2013
Published online: November 26, 2013

Abstract

AIM: To evaluate changes in neurotransmission induced by a psychoactive beverage ayahuasca in the hippocampus and amygdala of naive rats.

METHODS: The level of monoamines, their main metabolites and amino acid neurotransmitters concentrations were quantified using high performance liquid chromatography (HPLC). Four groups of rats were employed: saline-treated and rats receiving 250, 500 and 800 mg/kg of ayahuasca infusion (gavage). Animals were killed 40 min after drug ingestion and the structures stored at -80 ℃ until HPLC assay. The data from all groups were compared using Analysis of variance and Scheffé as post test and $P < 0.05$ was accepted as significant.

RESULTS: The results showed decreased concentrations of glycine (GLY) (0.13 ± 0.03 *vs* 0.29 ± 0.07, $P < 0.001$) and γ-aminobutyric acid (GABA) (1.07 ± 0.14 *vs* 1.73 ± 0.25, $P < 0.001$) in the amygdala of rats that received 500 of ayahuasca. Animals that ingested 800 mg/kg of ayahuasca also showed a reduction of GLY level (0.11 ± 0.01 *vs* 0.29 ± 0.07, $P < 0.001$) and GABA (0.98 ± 0.06 *vs* 1.73 ± 0.25, $P < 0.001$). In the hippocampus, increased GABA levels were found in rats that received all ayahuasca doses: 250 mg/kg (1.29 ± 0.19 *vs* 0.84 ± 0.21, $P < 0.05$); 500 mg/kg (2.23 ± 038 *vs* 084 ± 0.21, $P < 0.05$) and 800 mg/kg (1.98 ± 0.92 *vs* 0.84 ± 0.21, $P < 0.05$). In addition, an increased utilization rate of all monoamines was found in the amygdala after ayahuasca administration in doses: 250 mg/kg (noradrenaline: 0.16 ± 0.02 *vs* 0.36 ± 0.06, $P < 0.01$; dopamine: 0.39 ± 0.012 *vs* 2.39 ± 0.84, $P < 0.001$; serotonin: 1.02 ± 0.22 *vs* 4.04 ± 0.91, $P < 0.001$), 500 mg/kg (noradrenaline: 0.08 ± 0.02 *vs* 0.36 ± 0.06, $P < 0.001$; dopamine: 0.33 ± 0.19 *vs* 2.39 ± 0.84, $P < 0.001$; serotonin: 0.59 ± 0.08 *vs* 4.04 ± 0.91, $P < 0.001$) and 800 mg/kg (noradrenaline: 0.16 ± 0.04 *vs* 0.36 ± 0.06, $P < 0.001$; dopamine: 0.84 ± 0.65 *vs*

2.39 ± 0.84, $P < 0.05$; serotonin: 0.36 ± 0.02 *vs* 4.04 ± 0.91, $P < 0.001$).

CONCLUSION: Our data suggest increased release of inhibitory amino acids by the hippocampus and an increased utilization rate of monoamines by the amygdala after different doses of ayahuasca ingestion.

Key words: Ayahuasca; Amino Acids; Monoamines; Hippocampus; Amygdala

Core tip: Several studies have indicated that the main component of ayahuasca, *N*,*N*-dimethyltryptamine (DMT), is structurally similar to serotonin (5-hydroxytryptamine or 5-HT) and also has similarities with lysergic acid and mescaline, normally employed in drug addiction. This infusion contained DMT as a principal ingredient in a psychoactive beverage, used by more than 70 different indigenous groups spread throughout Brazil, Colombia, Peru, Venezuela and Ecuador. In human beings, it is also present in the brain as an endogenous substance and is found in blood, urine and cerebrospinal fluid. After oral administration of ayahuasca at different doses to naïve rats, we found that ayahuasca ingestion could modify neurotransmitter release in limbic brain structures.

Castro-Neto EF, Cunha RH, Silveira DX, Yonamine M, Gouveia TLF, Cavalheiro EA, Amado D, Naffah-Mazzacoratti MG. Changes in aminoacidergic and monoaminergic neurotransmission in the hippocampus and amygdala of rats after ayahuasca ingestion. *World J Biol Chem* 2013; 4(4): 141-147 Available from: URL: http://www.wjgnet.com/1949-8454/full/v4/i4/141.htm DOI: http://dx.doi.org/10.4331/wjbc.v4.i4.141

INTRODUCTION

Ayahuasca is a Quechua term derived from the juxtaposition of the words: (Aya) "soul", "dead spirit", and (Waska) "rope", "vine", and thus is loosely translatable as "vine of the souls" or "vine of the dead". Ayahuasca refers to the vine that is the principal ingredient of a psychoactive beverage used by more than 70 different indigenous groups spread throughout Brazil, Colombia, Peru, Venezuela, Ecuador[1] and North America[2]. The word ayahuasca is used to describe the spiritual force in the beverage, which usually also contains a combination of other plants, such as *Psychotria viridis* or *Diplopterys cabrerana*[3,4].

Several studies[5-8] have indicated that the main component of ayahuasca, *N*,*N*-dimethyltryptamine (DMT), is structurally similar to serotonin (5-hydroxytryptamine or 5-HT) and also has similarities with lysergic acid and mescaline. The beta-carbolines also present in ayahuasca include harmine (HRM), harmalina (HRL) and tetrahydroharmine (THH). DMT is a short-acting hallucinogenic tryptamine, which is present in several plants used as admixtures to the *Banisteriopsis caapi* (*B. caapi*) vine in ayahuasca preparations[8,9]. In human beings, it is also present in the brain as an endogenous substance and is found in blood, urine and cerebrospinal fluid[10]. DMT is psychoactive but is inactivated following oral administration, probably due to degradation by gastrointestinal and liver monoamine oxidase (MAO)[5,11,12]. However, when DMT is combined with inhibitors of MAO, such as the beta carbolines present in *B. caapi*, it becomes able to reach the systemic circulation and subsequently the central nervous system, thus producing its effects[5].

DMT acts as an agonist of serotonergic receptors and its association with MAO inhibition favors a still greater availability of 5-HT in the synaptic cleft. Moreover, THH inhibits reuptake of 5-HT as well as competing with DMT for the same receptors, 5-HT$_2$ and 5-HT$_{1A}$.

According to McKenna[7], a deficit of serotonin reuptake sites in the frontal cortex has been found to correlate with aggressive behavior in alcoholics. If THH is able to specifically reverse the deficit it might have clinical applications in the treatment of this disruptive behavior.

In this context, the present work was designed to analyze possible changes induced by ayahuasca to neurotransmission in the amygdala and hippocampus of rats, which received three different doses of this infusion by gavage.

For this purpose, monoamines (noradrenaline, NA; dopamine, DA; and serotonin, 5-HT) as well as their principal metabolites were quantified using high performance liquid chromatography (HPLC). The amino acid neurotransmitters glutamate, glycine (GLY), taurine (TAU) and gamma-aminobutyric acid (GABA) were also quantified in these structures. The main components of ayahuasca were measured by gas chromatography and the concentrations of DMT, HRL, HRM and THH were determined.

MATERIALS AND METHODS

Sample of ayahuasca

The infusion of ayahuasca was supplied by Professor Dr. Dartiu Xavier da Silveira, from the Psychiatry Department of Universidade Federal de São Paulo, and this infusion was prepared by the Núcleo Senhora Santana, Campo Grande, Brazil on May 22nd 2008, for Master José Roberto de Souza under the auspices of Centro Espírita Beneficente União do Vegetal, and in the care of C Otávio Castelo. The infusion was previously lyophilized and stored at -18 ℃ under vacuum. After this procedure, each 200 mL of infusion was converted in 40.8 g of powder, which was maintained under proper conditions.

Identification and quantification of ayahuasca components

The concentration of the main alkaloid ayahuasca components was determined in this work using a gas chromatography procedure, as previously reported[13]. Briefly, analyses were performed using an Agilent gas chromato-

graph equipped with a nitrogen-phosphorous detector (GC-NPD). Chromatographic separation was achieved on an HP ultra-2-fused-silica capillary column (25 m × 0.2 mm × 0.33 μm) film thickness using ultra-pure nitrogen as the carrier gas at a constant flow rate of 0.6 mL/min. Injections of 1 μL were made in split mode (ratio 1:20). The injector port and detector temperature were maintained at 200 and 250 ℃ respectively. The oven temperature was maintained at 150 ℃ for 1 min and programmed to rise at 10 ℃/min to 250 ℃ before being held at this latter temperature for 7 min.

A sample solution containing ayahuasca (0.5 mL), borate buffer (pH 9.0, 2.0 mL) and the internal standard diphenhydramine (100 μL of a solution 1.0 mg/mL) was loaded onto a C_{18} cartridge mounted on a vacuum manifold and conditioned with methanol (2 mL), deionized water (1.0 mL) and borate buffer (pH 9.0; 2.0 mL). The loaded cartridge was further washed with deionized water and with a solution of acetonitrile-water (1:9). After drying the cartridges under full vacuum for 7 min, the sample was eluted with methanol (2 mL). This solution (1 μL) was injected in the GC-NPD system and the retention time and concentration were obtained after comparison with stock standard solution[13].

Animal groups

At least 1 wk before the experiments, adult male Wistar rats, weighing 220-280 g, were randomly selected from the same pool and allocated to groups of five, housed under conditions of controlled temperature and humidity on a standard light/dark cycle of 12 h (lights off at 7:00 pm). Rat chow pellets and water were provided *ad libitum*. The experiments were performed with the approval of the Institutional Ethics Committee (DHEW Publication, NIH, 80-23), (number 1050/09) and all efforts were made to minimize animal suffering. Four groups of rats were employed: saline-treated (n = 5) and rats receiving 250 mg/kg (n = 8), 500 mg/kg (n = 8) and 800 mg/kg (n = 8) of lyophilized ayahuasca orally. The animals' behavior after drug administration was analyzed by three different observers and the rats were killed 40 min after drug ingestion. The brain structures were separated and stored at -80 ℃ until assay. Another group of rats (n = 8) was employed to study their behavior after drug administration (500 mg/kg). Changes in the behavior were analyzed by three different observers during 60 min.

Sample preparation and HPLC assay

Monoamines and amino acids were identified and quantified using a HPLC system with electrochemical and fluorescence detectors, respectively.

Brain structures were removed, placed on an ice-chilled plate, weighed and stored at -80 ℃ until assay. Tissues were ultrasonically homogenized in a 0.1 mol/L solution of $HClO_4$ containing 0.02% $Na_2S_2O_2$ (15 μL of solution for each milligram of tissue), dihydroxybenzylamine (DHBA, 146.5 ng/mL), as the internal standard for monoamines, and homoserine (HSER, 10 μg/mL), as the internal standard for amino acids. The samples were centrifuged at 11000 *g* at 4 ℃ for 40 min, and then the supernatant was filtered and injected into an HPLC system. The monoamines NA, DA and 5-HT, as well as their metabolites, were quantified as previously described by our group[14]. In summary, a Shimaszu LC-10AD isocratic system was employed, with a 20 μL injection loop and a Spheri-5 RP-18 5 μm column (220 × 4.6 mm), using electrochemical detection at 0.75 V and a mobile phase composed of phosphate/citrate pH 2.64, 0.02 mol/L, 0.12 mmol/L ethylene diamine tetraacetic acid and 0.06% heptane sulphonic acid, in 10% methanol, at a flow rate of 1 mL/min. Concentrations of monoamines and metabolites were expressed as mean ± SD ng/mg wet tissue. Turnover rates of monoamines were calculated by the ratio between metabolites and monoamine concentrations.

To assay amino acids, the supernatant was filtered and submitted to an o-phthaldehyde (OPA) derivatization and then injected into the HPLC system. Amino acid derivatization was done by dissolving 27 mg of OPA in 1 mL of methanol, adding 5 μL of 2-mercaptoethanol and 9 mL of 0.1 mol/L sodium tetraborate (pH 9.3) solution. Before sample analysis, a solution was prepared with 1 mL of stock solution and 2 mL of sodium tetraborate 0.1 mol/L. The pre-column derivatization was completed by reacting 100 μL of this solution with 50 μL of sample or amino acid standard solution for 2 min before the injection[15]. An isocratic HPLC system was used with a fluorescence detector, a 20 μL sample injector and an RP-18 column (50 × 4.6 mm). The mobile phase consisted of sodium phosphate 0.05 mol/L (pH 5.95) with methanol 11.5%. The flow rate of this HPLC system was 3.5 mL/min and the detector was employed with an excitation of 348 nm and emission of 460 nm.

Standard concentrations of amino acids and monoamines were tested and the retention time was verified for each substance to certify that there were no peaks overlapping on sample delivery. The amino acids were expressed as mean ± SD (nmol/L per milligram) wet tissue.

Statistical analysis

The data from all groups were compared using Analysis of variance and Scheffé as post test and $P < 0.05$ was accepted as significant.

RESULTS

Animal behavior

After ayahuasca administration, the behavior of the animals was evaluated qualitatively and compared with that of saline-treated rats. Ten min after infusion administration, all rats that received ayahuasca showed increased exploratory behavior, with increased sniffing and chewing. After this period, they exhibited hyperkinesia, oral and masticatory movements and blinking. The hyperkinesia evolved to loss of foot strength, enlarged base and semi-closed eyes, 30 min after ayahuasca administration. The number of fecal residues was similar in control and

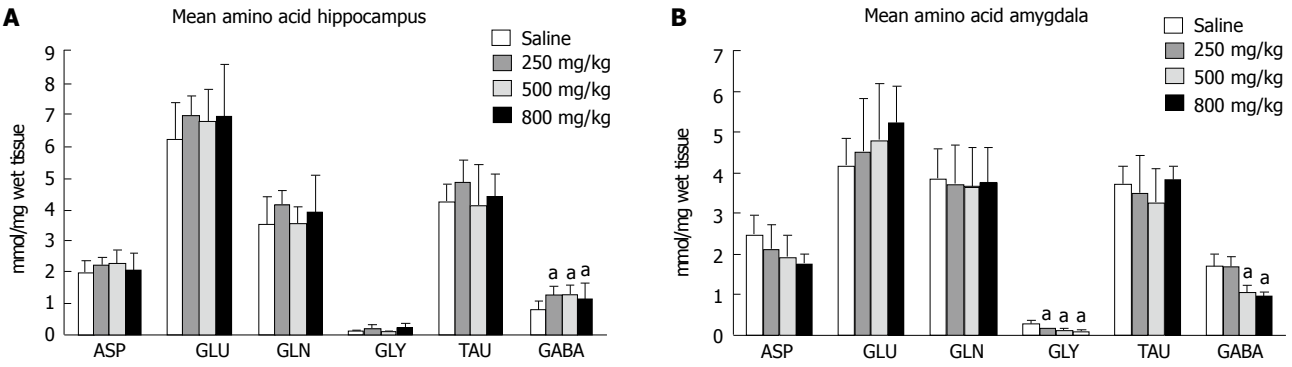

Figure 1 Alterations in amino acid concentrations in ayahuasca-treated rats. Hippocampus (A) and Amygdale (B). The bars represent mean ± SD of amino acid concentration. ASP: Aspartate; GLU: Glutamate; GLN: Glutamine; GLY: Glycine; TAU: Taurine; GABA: γ-aminobutyruc acid. Analysis of variance followed by Scheffé test and $^{a}P < 0.05$ *vs* saline.

Table 1 Concentration of each active principle measured by gas-chromatography

Ayahuasca lyophilized	250 mg/kg	500 mg/kg	800 mg/kg
DMT	6.02	12.04	19.26
THH	10.10	20.20	32.32
HRL	1.94	3.88	6.20
HRM	51.92	103.89	166.19

The concentrations of ayahuasca components present in the sample were quantified using Gas-Chromatography. DMT: *N,N*-dimethyltryptamine; THH: Tetrahydroharmine; HRL: Harmalina; HRM: Harmine.

treated animals. This altered behavior was progressively normalized and 60 min after ayahuasca administration, all rats showed normal activity.

The original infusion of ayahuasca employed contained DMT = 0.59 mg/mL, THH = 0.99 mg/mL, HRL = 0.19 mg/mL and HRM = 5.09 mg/mL. Table 1 summarizes the concentration of each active principle administered to each group of rats.

Changes in amino acid content in the amygdala and hippocampus

When the concentrations of amino acids were analyzed in the hippocampus, we found an increased level of GABA in the groups of rats that received 250, 500 and 800 mg/kg of ayahuasca, when compared with saline-treated animals (Figure 1A). In contrast, the data illustrated in Figure 1B show that the amygdala presented a decreased concentration of GLY in all drug-treated groups and decreased GABA only in those rats that received the highest ayahuasca concentrations (500 and 800 mg/kg).

Changes in concentrations of monoamines and their metabolites

The hippocampus of ayahuasca-treated rats showed greater variation when 500 and 800 mg/kg of drug was administered. Animals that received these doses showed increased concentrations of 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid and homovanillic acid (data not shown). In addition, the level of 5-HT was also increased in those rats that received 500 or 800 mg/kg of ayahuasca (Figure 2A). However, the comparison between drug-treated and saline-treated groups showed only a decreased utilization rate for 5-HT in the hippocampus of rats that received 800 mg/kg (Figure 3A).

The amygdala presented the biggest change with regard to monoamine levels. NA, DA and 5-HT all showed increased concentrations in all the studied groups. Analyzing the utilization rates of monoamines in the amygdala, we found that NA, DA and 5-HT were less utilized or less degraded, as shown in Figure 3B.

DISCUSSION

Ayahuasca infusions prepared in different South American countries contain different concentrations of psychotropic agents. According to McKenna *et al*[5], each milliliter of ayahuasca from Peru contains DMT (0.6 mg), HRM (4.67 mg), HRL (0.41 mg) and THH (1.6 mg). Meanwhile, the great majority of Brazilian ayahuasca contains, on average: DMT (0.6 mg/mL); HRM (1.2 mg/mL) HRL (0.2 mg/mL) and THH (1.07 mg/mL)[16]. In this context, our data are in accordance with those described by other authors since we found DMT = 0.59 mg/mL, THH = 0.99 mg/mL, HRL = 0.19 mg/mL and HRM = 5.09 mg/mL in the ayahuasca sample.

The present study aimed to investigate the effects of the ingestion of different concentrations of the ayahuasca infusion upon the levels of some neurotransmitters (monoamines and amino acids), as well as the utilization rate of monoamines in the hippocampus and amygdala of naive rats.

No changes were found in the glutamate concentrations, either in the hippocampus or the amygdala of treated animals, which indicates that this excitatory amino acid is not involved in ayahuasca-induced behavioral changes. However, a reduction in GLY levels in the amygdala was observed with the administration of 250, 500 and 800 mg/kg of infusion, and a reduction in GABA levels was seen after administration of 500 and 800 mg/kg, suggesting increased release of both inhibitory amino acids in this brain structure. In contrast, in the hippocampus,

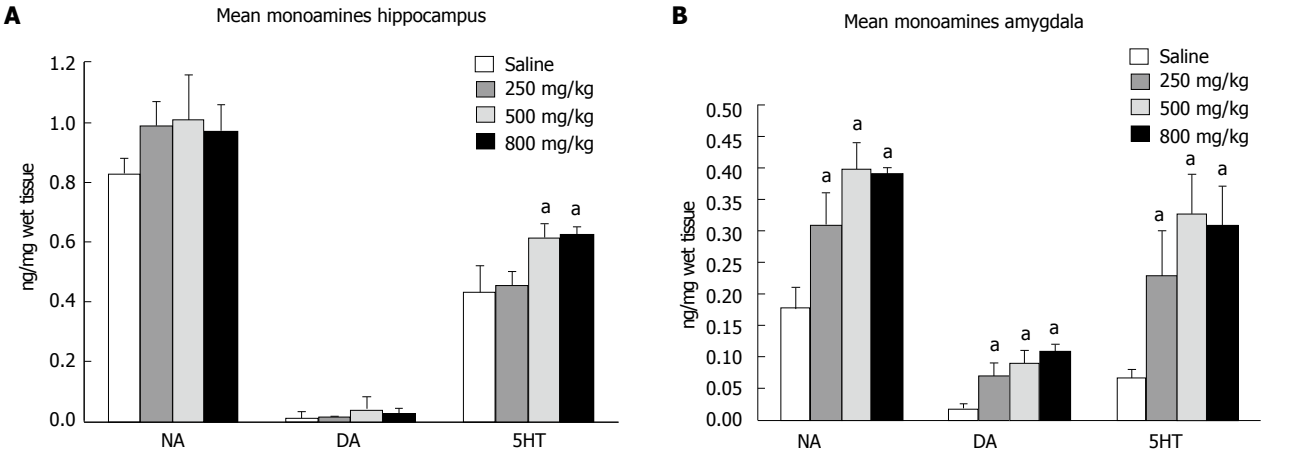

Figure 2 Alterations in monoamine concentrations of ayahuasca-treated rats. Hippocampus (A) and Amygdale (B). The bars represent mean ± SD. Amino acid concentration: NA (NE): Norepinephrine; DA: Dopamine; 5HT: 5-hydroxytryptamine. Analysis of variance followed by Scheffe test and $^{a}P < 0.05$ *vs* saline.

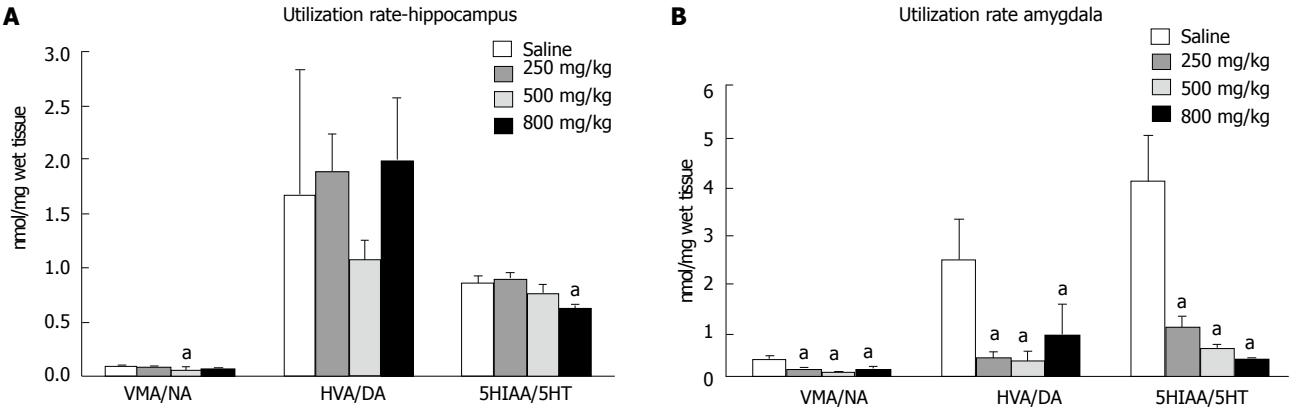

Figure 3 Utilization rate of monoamines. Hippocampus (A) and Amygdale (B). The utilization rate was estimated as a ratio between metabolite/ neurotransmitter concentrations: VMA: 4-hydroxy-3-methoxy mandelic acid; NA: Norepinephrine; DA: Dopamine; 5HIAA: 5-hydroxyindoleacetic acid; HVA: 4-hydroxy-3-methoxyphenylacetic acid; 5HT: 5-hydroxytryptamine. The bars represent mean ± SD of ratio. The results were evaluated by Analysis of variance followed by Scheffé test. ($^{a}P < 0.05$ *vs* saline).

an increased concentration of GABA was found in all ayahuasca-treated groups, suggesting decreased release of this neurotransmitter, compatible with increased excitation in the hippocampus and increased inhibition in the amygdala of ayahuasca-treated rats.

In this context, we showed here, for the first time, that ayahuasca induces changes in the concentration of inhibitory amino acids in two limbic structures. The opposite effects found in the hippocampus and amygdala could support the activation and/or inhibition of several pathways involved in important processes, such as memory and learning and emotional behavior. Furthermore, the GABA ergic system has also been linked to an amnesic function in the hippocampal-dependent declarative memory[17] and a change in this pathway could represent modification of this function. In direct opposition to this idea, data from Doering-Silveira *et al*[18] showed that ayahuasca-user adolescents did not differ from control subjects when neuropsychological tests were applied to both groups. Thus, more studies are needed to elucidate these findings.

The determination of monoamine content showed that among the analyzed structures, the amygdala was most affected by ayahuasca treatment; all the concentrations used in this work increased the levels of NA, DA and 5-HT in this limbic structure. The utilization rates of these monoamines were also reduced, suggesting MAO inhibition or increased synthesis of these neurotransmitters, or both.

In the hippocampus, 5-HT levels were also increased in rats that received 500 and 800 mg/kg of ayahuasca. However, the comparison between drug-treated and saline-treated groups showed a decreased utilization rate for 5-HT in the hippocampus only when the higher dose of ayahuasca was employed.

These data show that, although with some variation, ayahuasca increases the concentration of monoamines in limbic structures, mainly in the amygdala. Furthermore, previous studies have shown an increased stimulation of 5-HT receptors by the individual ayahuasca components [5,6] showing an over stimulation of monoamine pathways in the brain.

According to Schwarz *et al*[19], HRM and HRL also stimulate DA release from striatal slices and these data, in

association with MAOA inhibition, suggest that *B. caapi* could be tested in the treatment of Parkinson disease. In addition, Schmoldt *et al*[20] observed that MAO inhibition is able to reduce oral ethanol self-administration, due to high levels of DA and 5-HT in the synaptic cleft. In this context, ayahuasca could also be useful in the treatment of alcohol dependence. Recent data showed an important application in therapies for addiction[9,11,21], anxiety disorders[22] and Parkinson disease[19,23,24], and modulates REM and slow-wave sleep in healthy volunteers[25]. HRM is also related to inhibition of angiogenesis and suppression of tumor growth through activation of p53 in endothelial cells[26].

Taken together, these data show that ayahuasca components have different actions on different brain structures, involving changes in monoamines and amino acid concentrations.

COMMENTS

Background

Ayahuasca is an infusion containing psychoactive compounds, such as *N,N*-dimethyltryptamine (DMT), is structurally similar to serotonin (5-hydroxytryptamine) and also has similarities with lysergic acid and mescaline. This infusion also has beta-carbolines, such as harmine, harmalina and tetrahydroharmine. DMT is a short-acting hallucinogenic tryptamine, which is present in several plants used as admixtures to the *Banisteriopsis caapi* vine in ayahuasca preparations.

Research frontiers

As ayahuasca use has been growing in the world, mainly in South and recently in North America, the knowledge of how it can modify normal neurotransmitters in limbic structures is important since this infusion could be employed in the treatment of other brain pathologies.

Innovations and breakthroughs

This work shows, for the first time, detailed changes in amino acids and monoamines, as well as their utilization rates in limbic structures of the central nervous system, after ayahuasca ingestion by naïve rats.

Applications

Clinical use of ayahuasca could be important as an additional drug in treatment of major depression and/or in other central pathologies.

Terminology

Utilization rate of a monoamine is used to visualize the release of this neurotransmitter in specific regions of the CNS. It is described as the rate between its main metabolite and neurotransmitter concentration (metabolite concentration/monoamine concentration).

Peer review

Ayahuasca is a psychoactive plant preparation which contains a serotonin analog dimethyltryptamine and monoamine oxidase beta-carbolines. However, *in vivo* effects on monoamine neurotransmitters and other amino acid transmitters have not been reported. In this manuscript, the authors observed the effect of orally administered ayahuasca on the content of monoamines (and their metabolites) and amino acid neurotransmitters in two important brain structures in the limbic system. Their results demonstrated that ayahuasca mainly increased monoamines in the amygdala, accompanied by a reduction of their metabolites.

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P- Reviewers: Chang YC, Lei S **S- Editor:** Zhai HH
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