Maastricht University

Kinetics and effects of orally administered ATP

Citation for published version (APA):

Coolen, J. C. M. (2011). *Kinetics and effects of orally administered ATP*. [Doctoral Thesis, Maastricht University]. s.n.]. https://doi.org/10.26481/dis.20110511jc

Document status and date:
Published: 01/01/2011

DOI:
10.26481/dis.20110511jc

Document Version:
Publisher's PDF, also known as Version of record

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
• The final author version and the galley proof are versions of the publication after peer review.
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Kinetics and effects of orally administered ATP

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This PhD project was supported by the Graduate School VLAG (Food Technology, Agrobiology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences. The studies presented in this thesis were conducted at the Department of Epidemiology and the Department of Pharmacology & Toxicology, Maastricht University, Nutrition and Toxicology Research Institute Maastricht, The Netherlands, in close collaboration with the Laboratory for Physical Chemistry and Colloid Science, Wageningen University.

Kinetics and effects of orally administered ATP
Thesis, Maastricht University, Maastricht, The Netherlands

ISBN 978 90 86595501

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Design and layout: Erik Coolen
Printed by: Drukkerij Wilco

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Financial support for printing of this thesis has kindly been provided by
'Fagron BV',
‘Greiner Bio-one’ and
‘Sanquin Blood Supply, Unit Reagents.’

Kinetics and effects of orally administered ATP

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,

op gezag van de Rector Magnificus,

Prof. mr. G.P.M.F. Mols,

volgens het besluit van het College van Decanen,

in het openbaar te verdedigen

op woensdag 11 mei 2011 om 14.00 uur

door

Johannes Cornelis Maria Coolen

Geboren op 26 april 1981 te Tilburg

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Chapter 1

General Introduction

Nucleotides

Nucleotides are compounds that consist of a base, a sugar (either ribose or deoxyribose), and one to three phosphate groups. They have numerous essential functions in the mammalian body.

Nucleotides form the building blocks of DNA and RNA (figure 1), making them vital for encoding all genetic information in the human body. Nucleotides are also involved in metabolism, cellular signalling (both intra- and extracellular) and in various cofactors of enzymatic reactions, as will be exemplified below.

One of the best known nucleotides is adenosine triphosphate (ATP) (figure 1). It can be produced endogenously from glucose through glycolysis, the citric acid cycle, and oxidative phosphorylation in mitochondria.

Once ATP is produced in the mitochondria, it is transported across the outer mitochondrial membrane, primarily by voltage-dependent anion channels (VDAC)[(1)]. ATP can also be salvaged after it is released from cells or it can come from an exogenous dietary source. The concentration in the cell ranges from 1-10 mM. ATP plays a key role as a carrier of chemical energy. The bounds between phosphate groups are responsible for the high energy content of ATP. The energy that is released upon hydrolysis of ATP to ADP by adenosine dehydrogenase ($\Delta G° =$ -30,5 kJ/mol) can for instance be used for synthesis of organic molecules and transmembrane transport through ATP-binding cassette transporters (ABC-transporters).

The use of ATP as a carrier of chemical energy is very common in eukaryotes. The incorporation of mitochondria into eukaryotic cells is believed to have occurred very early in evolution, since it provided cells with the possibility of aerobic respiration with ATP as chemical energy carrier[(2)].

Next to playing a role in chemical energy supply, ATP is also involved in intracellular signal transduction, for instance after it is converted to cyclic AMP (cAMP) by the enzyme adenylyl cyclase, located on the inner side of the plasma membrane[(3)]. Also several cofactors are derived from ATP, like coenzyme A (CoA), and the electron acceptors flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD), both of which can be used to transfer electrons from one molecule to another[(4)].

Besides its intracellular functions, ATP is also well known for its extracellular functions. Although the concentrations outside the cell are normally below the micromolar range (between 10 and 100 nM[(5)]), extracellular ATP influences many biological processes, including neurotransmission, muscle contraction, inflammation and cardiac function[(6, 7)]. Whereas intracellular concentrations are maintained at high levels, ATP released from cells into the extracellular compartment is tightly regulated and its concentration is kept very low by ecto-enzymes[(8-10)].

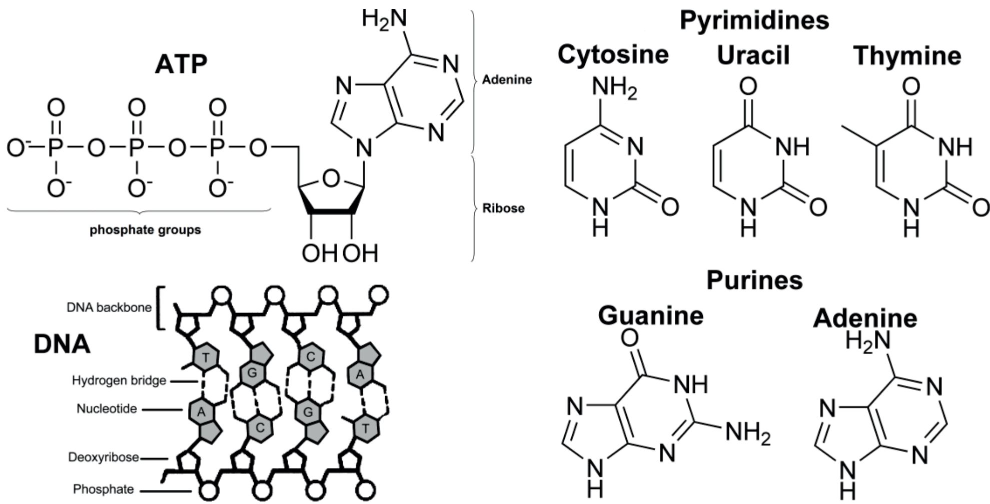

Figure 1: Nucleotides consist of a nitrogenous base, a pentose sugar, and one or more phosphate groups. The nitrogenous base is either a purine or a pyrimidine. Pyrimidine bases are six-membered rings, and include uracil, cytosine (C) and thymine (T). Purine bases have a second five-membered ring, and include adenine (A), guanine (G), hypoxanthine and xanthine. A purine or pyrimidine base linked to a pentose molecule constitutes a nucleoside. A nucleotide is a phosphate ester of a nucleoside, and may occur in the mono, di- or triphosphate form. The pentose is either ribose or deoxyribose; the ribonucleotides and deoryribosenucleotides serve as the monomeric units of RNA and DNA, respectively.

Extracellular ATP degradation

Ecto-enzymes are divided over four families. Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) are the products that are formed when one and two phosphates are cleaved off, respectively, by ectonucleoside triphosphate diphosphohydrolases (NTPDases) (figure 2)(11). Next, ectonucleotide pyrophosphate/phosphodiesterase (NPP) catalyzes the hydrolysis of ADP to AMP and of AMP to adenosine. Alkaline phosphatases (AP) can catalyze the hydrolysis of ATP, ADP and AMP. The degradation of AMP to adenosine is also done by ecto-5'-nucleotidase (CD73)(12). Adenosine can be broken down further to inosine by adenosine deaminase (ADA) or to adenine by a purine nucleoside phosphorylase. In the final stages of purine metabolism, the enzyme xanthine oxidase (XO) is responsible for the degradation of hypoxanthine and xanthine to the end-product uric acid(13).

Functions of extracellular purinergic metabolites

All of the various degradation products have their own functions, because they are ligands for different membrane-bound purinergic receptors. Transient increases in extracellular ATP are vital for cell-to-cell communication in nervous,

vascular and immune systems[14, 15]. In damaged tissues, ATP is a natural endogenous adjuvant which is released from activated immune cells[16], macrophages[17], microglia[18], and platelets[19] that initiates inflammation, and further amplifies and sustains cell-mediated immunity through P2 receptor-mediated purinergic signalling[20].

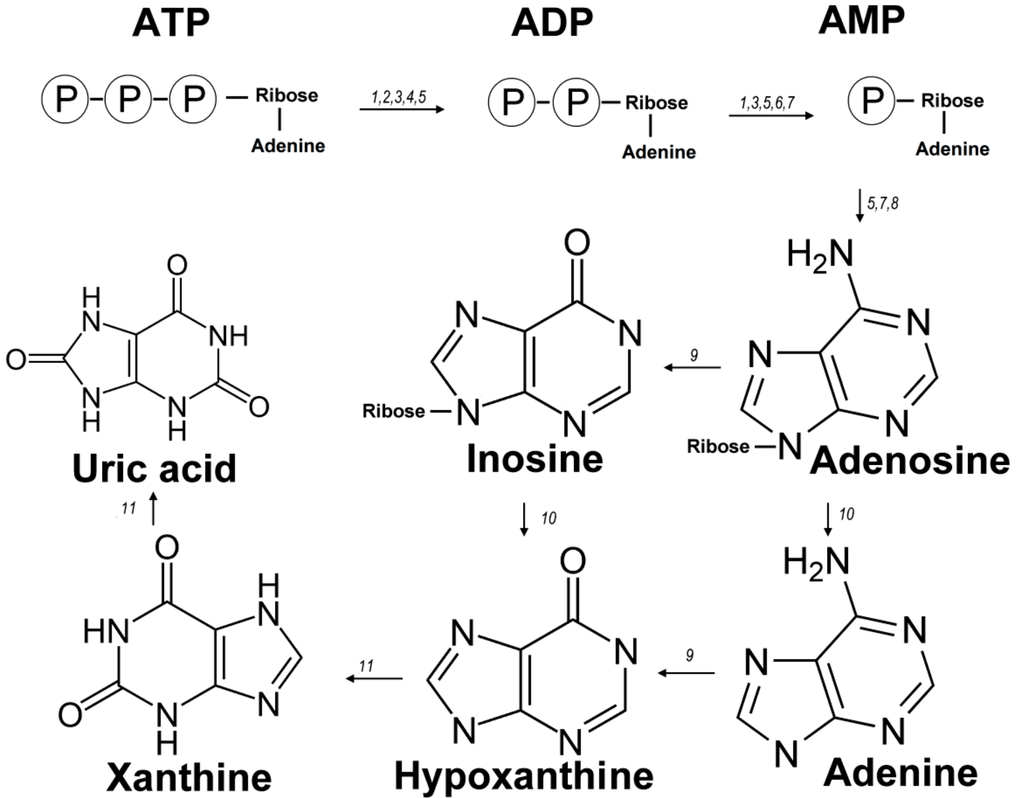

Figure 2: Metabolism of ATP to uric acid. Numbers in italics represent: 1, ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) 1; 2, NTPDase 2; 3, NTPDase 3; 4, NTPDase 8; 5, alkaline phosphtase; 6, ectonucleotide pyrophosphatase/phosphodiesterase (NPP) 1; 7, NPP2; 8, ecto-5'-nucleotidase (CD73); 9, Adenosine deaminase (ADA); 10, purine nucleoside phosphorylase (PNP) and 11, xanthine osidase (XO).

Purinergic receptors

Purinergic signalling is rapidly becoming recognised as essential for the regulation of tissue and organ function. Drury & Szent Györgyi discovered the vasodilatory effect of extracellular purines in 1929[21]. First, observational studies helped to develop theories on purinergic signalling. Later, Geoffrey Burnstock proposed extracellular ATP to be a neurotransmitter conducting noradrenergic and noncholinergic neurotransmission of the gut and urinary bladder in 1972[22]. Now, purinergic receptors have been detected in virtually every type of cell in every type of tissue, leading to an exponential increase in the number of articles on purines[23]. Purinergic signalling was found to have appeared early in evolution[24] and to be

widespread in most non-neuronal and neuronal cell types[25]. Also the recognition that there is both long-term purinergic signalling in cell-proliferation, differentiation, development, and regeneration and short-term purinergic signalling in neurotransmission and secretion[26, 27] has helped the field of purinergic signalling to establish itself as an important part of various signalling routes.

Purinergic receptors are classified in two families, namely P1 and P2 receptors. P2 receptors have ATP and other nucleotides as their primary ligands, whereas adenosine is the ligand for P1 receptors. The P2 family can be divided into two subfamilies, P2Y and P2X[25]. P2Y receptors are G protein-coupled receptors, of which twelve subtypes have been described ($P2Y_1$. $P2Y_2$, $P2Y_{4\text{-}6}$ and $P2Y_{8\text{-}14}$[28-30]. P2X receptors are ATP-gated ion channels of which seven subtypes have been identified ($P2X_{1\text{-}7}$)[31-33]. P1 receptors are G-protein coupled receptors which are activated by adenosine and are subdivided into A_1, A_{2A}, A_{2B} and A_3 receptor subtypes[34, 35]. Signal transduction through P2Y receptors occurs via Gq proteins activating phospholipase C (PLC), which for most receptors induces the release of inositol triphosphate (IP_3) and thereby results in mobilization of Ca^{2+} from intracellular stores[36].

When P2X receptors are activated by ATP, sodium will be transported into the cell, whereas potassium and calcium will be transported out of the cell through the formed ion channel. This results in activation of various signalling molecules[37], such as extracellular-signal regulated kinase (ERK), which in turn stimulates transcription of NFκB[38]. NFκB is a main contributor to the synthesis and secretion of cytokines and is associated with the development of several diseases, such as inflammatory arthritis, lung fibrosis, cancer, diabetes and stroke[39].

When LPS-stimulated monocyte-derived dendritic cells are constitutively exposed to low ATP concentrations (100-200 μM), this will lead to inhibition of the cytokines IL-12, TNF-α, IL-1ß, and IL-6[40]; P2Y11 receptors are thought to participate in this reaction. Also other in vitro studies, using for instance macrophages, have demonstrated the inhibition of inflammatory cytokine release[5, 41, 42]. In whole blood, addition of ATP leads to reduction in release of TNF-α and an increase in IL-10, thus inhibiting inflammation[43, 44].

Intravenous ATP administration

Several human intervention trials with advance stage cancer patients have been performed to investigate whether ATP infusion can exert favourable effects. The main clinical symptoms that were investigated were those that are associated with the cancer cachexia syndrome: decreased food intake, progressive involuntary weight loss with depletion of lean body mass, and an impaired immune response[45]. Cancer-cachexia syndrome has a prevalence of up to 80% in patients with advanced cancer[46]. The reported beneficial effects of ATP infusion ranged from the prevention of deterioration of muscle mass, functional performance and

weight[47, 48], to lowering pain and increasing life expectancy[49]. In a randomized clinical trial conducted by Agteresch et al.[47, 48, 50], patients with stage IIIb/IV non-small cell lung carcinoma (NSCLC) received ATP, up to 75 µg/kg/min (as tolerated) for 30 hours intravenously at 2- to 4-week intervals. Increased concentrations of ATP were observed in the blood plasma of these patients following intravenous infusion. Leij-Halfwerk et al.[51] reported that the ATP concentrations in the livers of patients with NSCLC were initially lower than those of healthy volunteers, but recovered back to normal concentrations after intravenous ATP infusions.

Intravenous infusion is, however, quite a burden in particular for patients with advanced cancer, but also the need for medical supervision and the accompanying high costs are important drawbacks of the intravenous administration method. Moreover, intravenous administration is limited to infusion rates of approximately 75-100 µg/kg/min to avoid the occurrence of side effects[47, 52]. As an alternative to infusion, the oral administration of ATP might yield similar effectiveness, but less side-effects.

Oral route of administration of ATP

ATP is present in substantial concentrations in a number of foods (e.g. meat, soy, mushroom) and in breast milk. In breast milk, nucleotides are reported to enhance the gastrointestinal and immune systems[53-55]. Types of food that are rich sources of dietary nucleotides include organ meat like liver, heart and kidney, fresh seafood, beer, and some types of beans[54]. The amount of nucleotides in an average meal is by far insufficient to represent the amount that is needed in the body. In a purine-restricted diet, purines are often restricted to 100-150 milligrams per day. In normal diets, the amount of nucleotides ingested will be between 500 and 1000 mg/day, but they can reach up to about 5000 mg/day. When ATP is ingested with food, there are a number of challenges it has to face before being of use to the human body. First, the large molecular weight, the negative charge at physiological pH (pH 7.4), and the lack of known nucleotide transporters makes both passive and active transport of intact ATP over outer cell membranes highly unlikely. Second, ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) present on the luminal side of enterocytes will dephosphorylate ATP via ADP to AMP. AMP, in turn, will be subject to further degradation as ecto-5'-nucleotidase (CD73) and alkaline phosphatase will degrade it to adenosine[56]. For the effective use of ATP, ATP does not need to be absorbed intact, since adenosine can be taken up into the enterocytes of the intestinal wall. This occurs through concentrative (CNT) or equilibrative (ENT) nucleoside transporters[57] on the baso-lateral side of enterocytes. Provided that adenosine is released intact into the vascular bed, adenosine will come into contact with erythrocytes, which will take up adenosine through ENTs in their membranes[58, 59]. In vivo studies in animals and humans

have shown that adenosine can be used for the synthesis of ATP inside erythrocytes.

In humans, there has been no thorough investigation as to whether adenosine originating from intestinal uptake can also be used for ATP production in erythrocytes. Given adenosine's own role as an important extracellular signaling molecule (it facilitates a variety of physiological responses, including coronary vasodilation, neuromodulation, and platelet aggregation), its concentration is tightly regulated[60]. Moreover, after adenosine is absorbed from the intestine and released into the portal vein, it will quickly enter the liver. In the liver, adenosine will be broken down to uric acid by the enzymes adenosine deaminase and xanthine oxidase[61].

Although the evidence of effectiveness of oral ATP administration in sports and fitness is not quite solid, commercial marketing of ATP as an aid in in particular the bodybuilding area of sports is widespread[62, 63].

Evidence from animal studies by Kichenin et al. shows that in rats some of the molecules from which ATP can be regenerated can be absorbed from the intestinal lumen and secreted into the portal vein[64, 65]. ATP, adenine, inosine, adenosine, AMP, ADP and uric acid concentrations in plasma from the portal vein were increased when, during a surgical procedure, an isolated part of the jejunum was injected with ATP. This occurred specifically in rats that were treated with ATP (10 mg/kg/day) for 30 days.

When considering the oral route as an alternative to ATP infusion, some of the potential limiting factors, like ATP metabolism in the enterocyte, need to be addressed. One potential way to avoid metabolism of ATP in the upper gastrointestinal tract, is to let ATP evade the enzymes and acidic environment in the stomach that may break it down. An enteric coating would bypass the stomach with its digestive enzymes and let ATP reach the intestine, where it could be absorbed intact. Besides enzymes, the acidic environment of the stomach might also cause the breakdown of ATP, since ATP's catalytic phosphate (the phosphate located furthest away from adenine and which is cleaved off during enzyme-catalyzed hydrolysis) is acid-labile[66]. The gastrointestinal tract is pictured schematically in figure 3.

One possible use of an enteric coating is to target a specific area along the gastrointestinal tract. In case of ATP, it is for example known that the proximal small intestine of mice exhibits higher ATPase activity compared to the distal small intestine[67]. By protecting ATP by a coating consisting of polymers with different pH solubility, the resulting release of ATP in specific areas of the small intestine could therefore result in differences in uptake efficiency. This technique employs the increasing pH along the gastrointestinal tract (figure 3).

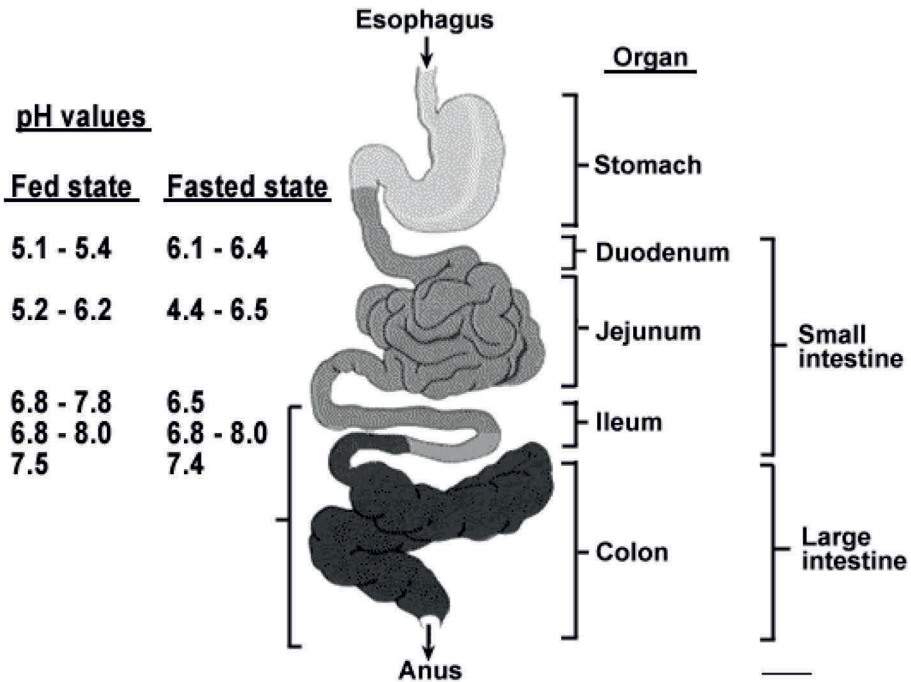

Figure 3: A schematic drawing of the human gastrointestinal tract. Using enteric coating, specific parts of the gastrointestinal tract can be targeted for release of compounds that would otherwise be degraded by the acidic environment of the stomach or by enzymes that are present in certain parts of the gastrointestinal tract. pH-dependent enteric coating depend on the changes in pH that occur along the gastrointestinal tract. The pH values for the fasted and fed state are taken from Gray and Dressman (1996)[97].

There has been a limited number of studies on oral ATP administration in humans. In a 14-day study intended to investigate anaerobic performance, Jordan et al.[(68)] administered an enterically coated oral ATP supplement containing dosages of up to 225 mg daily. These authors observed small increases in performance of high-intensity exercise bouts of very short duration, but no statistically significant change of whole blood or plasma ATP concentrations. The authors suggested that either the molecular size of ATP might prevent absorption, or that ATP might be dephosphorylated intraluminally to adenosine. A placebo-controlled RCT was designed to investigate the efficacy of daily administration of 90 mg ATP for 30 days on subacute low-back pain[(69, 70)]. Results showed that patients in the ATP group took significantly fewer analgesics than patients in the placebo group. Also within the department of Epidmiology of Maastricht University some pilot-studies were performed in small subject-numbers to investigate the effects of oral ATP administration when ATP was ingested either dissolved in water or as enteric coated capsules[(71)].

These pilot studies suggested a possible rise in blood uric acid concentration, but not ATP after oral ATP administration[(71)]. Explanations for the increased uric acid concentrations included the metabolism of unprotected ATP dissolved in water in the stomach, and the premature release of ATP from enteric coated 00-size cap-

sules due to inadequate coating properties. These uncertainties called for a more thorough and better controlled investigation of oral ATP administration. The next paragraph will cover the characteristics of uric acid and its diverse functions.

Plasma uric acid

Humans and higher primates have much higher plasma uric acid concentrations than other species. This probably arose from parallel, independent mutations of the gene coding for uricase in two lineages leading to higher primates in the Miocene (5-23 million years ago)[72]. This caused uric acid to be the final enzymatic endproduct in purine metabolism, and not the more soluble and readily excreted allantoin[73]. The fact that these mutations were able to maintain themselves, strongly suggests that there must have been a selection advantage to having higher plasma uric acid levels[73]. Several hypotheses have been put forward.

Advantages of high plasma uric acid concentrations

The first and most quoted hypothesis involves the important role uric acid plays as an antioxidant in plasma[74]. Proctor[75] and later Ames[74] suggested that the uric acid mutation may have benefited survival because it maintained adequate plasma antioxidant activity after the loss of ascorbate (vitamin C) synthesis due to an earlier mutation[76]. Consistent with its antioxidant function, uric acid has been suggested to be protective in various conditions, such as acute stroke, multiple sclerosis, and Parkinson's disease[77].

A second hypothesis focuses on uric acid's role in innate immunity. Specifically, uric acid may aid in the immune recognition of dying cells[78], help activate the inflammasome which is responsible for interleukin-1 beta (IL-1ß) release[79], and support the immune rejection of tumor cells[80]. More recently, uric acid release from cells in the area directly surrounding a site of vaccin injection was found to aid the effectiveness of vaccination[81, 82].

A third hypothesis links the higher uric acid concentrations to a survival advantage in early primates by helping to maintain blood pressure during periods of dietary change and environmental stress[83]. Uric acid has also been proposed to have neurostimulant properties based on its similarity in chemical structure with caffeine[84] and due to studies suggesting it may have a role in increasing mental performance[85].

Disadvantages of high plasma uric acid concentrations

Although mainly positive properties are ascribed to uric acid when it is present at normal plasma concentrations of 175-355 μmol/L, uric acid can form deposits of monosodium urate (MSU) crystals when plasma concentrations are above 420 μmol/L for prolonged periods of time. These crystals are the causative agent of gout, which affects about 1% of the population, the majority being male[86]. Gout is a painful inflammation of the joints, which may affect surrounding tissues and skin. In most cases, patients are hyperuricemic (>420 μmol/L), and a Western diet (a diet high in fat, fructose and protein) and obesity are often associated with this condition[87, 88]. The prevalence of gout and hyperuricemia has increased in the last century[89]. Gout is a condition exclusive to primates, because all other species are able to further break down uric acid to allantoin, which is far more soluble and readily excreted through the kidneys[90]. Although more than 10% of the people with a predominantly Western diet have elevated circulating uric acid concentrations, only a minority of hyperuricemic patients develop gout, suggesting that other factors are involved in the pathogenesis of gout.

Acute attacks of gout are often triggered by trauma, surgery, intercurrent illness, excess alcohol intake or drugs that alter serum uric acid levels. Such events may stimulate de novo generation of MSU crystals (e.g. derived from dying cells) or may trigger their release from preformed MSU crystal deposits within the joint[91]. In this manner, MSU crystals can act as a 'danger signal', resembling exogenous stimuli (e.g. microbial LPS) that activate the innate immune system, often the first line of defence protecting the host from invading microbial pathogens[78, 92].

The innate immune system may play a crucial role in triggering MSU crystal-induced inflammation. For instance, MSU crystals were demonstrated to stimulate synovial cells, monocyte-macrophages, and neutrophils to produce TNF-α, IL-8, IL-1β, IL-6 and monocyte chemotactic factors, which, in turn, induce acute inflammation[93]. Macrophage activation by MSU crystals was reported to require the recognition of MSU crystals by Toll-like receptors (TLR) 2 and 4, after which IL-1β production is stimulated via the downstream TLR adapter protein myeloid differentiation factor 88 (MyD88)[94]. The importance of neutrophils in acute gout is supported by observations that the injection of MSU crystals into the joints of animal models, which normally reproduces the symptoms of gout, gives a markedly attenuated response when neutrophils are depleted[95]. The neutrophils that are attracted to the joint actively phagocytose MSU crystals, resulting in membranolysis, generation of reactive oxygen species and in release of lysosomal enzymes, prostaglandin E2, leukotrienes, and IL-1[96].

Aims of the thesis

As indicated in this introduction, both intracellular and extracellular ATP are important in human physiology. ATP has been successfully administered intravenously to cancer patients in randomized clinical trials, resulting in several favourable effects, such as reducing pain and increasing life expectancy. The intravenous route of administration of ATP is, however, a considerable burden for advanced stage cancer patients. Therefore, the aim of the work described in this thesis was to evaluate whether ATP can be effectively administered orally.

At the start of this project, there was no analytical method available that could quickly and reliably measure ATP and its metabolites in a single run. Therefore, we developed an HPLC method for measuring ATP and seven of its metabolites in human blood samples and optimized it for quick analysis in one chromatographic run. This is described in **chapter 2**. This method was applied to investigate ATP metabolism in human blood in an ex vivo setting and subsequently used for measuring ATP metabolites in blood samples collected in the human intervention studies described in chapter 3, 4, and 5.

A comparative study using two different oral administration routes of ATP is described in **chapter 3**. In eight volunteers, 5000 mg ATP was either administered dissolved in water directly in the duodenum through a nasoduodenal tube or by means of enteric coated pellets. Two variants of the pellets were tested next to each other, each targeting a different section of the small intestine. The study was blinded and placebo-controlled and between each single administration of ATP was a period of one week. In this study we measured blood samples collected during a period up to 8 hours after administration.

Elaborating on the work presented in chapter 3, a study using a prolonged period of ATP administration is described in **chapter 4**. This time, 4 groups of eight volunteers take in a daily dosage of ATP ranging from 0, 250, 1250 to 5000 mg. The enteric coated pellets that gave optimal results in the previous study were used. On the first and last day of the four-week study period, all volunteers received a single dose of 5000 mg ATP. The difference in blood ATP and metabolite concentrations between the first and last day were compared in order to gain more insight into the effects of a prolonged period of daily oral ATP administration.

Chapter 5 describes an ex vivo study with LPS-PHA stimulated whole blood. In this study we looked at the immunomodulatory effects of a 24-hour incubation period with ATP. In order to investigate the effects of ADP, AMP, adenosine, inosine, hypoxanthine and uric acid, the blood was also incubated with these metabolites of ATP. At different time-points after LPS-PHA stimulation several immunomodulatory markers were studied. Also the involvement of the transcription factor NFκB has been investigated.

In **chapter 6**, an ex vivo study using fresh blood from healthy volunteers is described to further investigate the effects of uric acid on inflammation in blood ex vivo. Blood was incubated in the presence of lipopolysaccharide (LPS), phytohae-

magglutinin (PHA) and either uric acid or monosodium urate (MSU) crystals. We looked at the production of a panel of 17 different cytokines after an incubation period of 24 hours. Besides this investigation into the inflammatory effects, also the antioxidant capacity was investigated.

In **chapter 7**, the findings described in the previous chapters will be discussed. Some further prospects and future perspectives will be presented.

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Chapter 2

Simultaneous determination of adenosine triphosphate and its metabolites in human whole blood by RP-HPLC and UV-detection

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J Chromatogr B Biomed Appl. 2008 864:43-51.

Abstract

To obtain insight in mechanisms of action of extracellular ATP and adenosine, a simple HPLC method has been optimized and applied to investigate ATP metabolism in human whole blood ex vivo. This method provided good chromatographic resolution and peak shape for all eight compounds within a 19 min run time. The baseline was clean, the lower limit of quantification was below 0.3 µmol/L for all adenine nucleotides and the method demonstrated good linearity. Within-day precision ranged from 0.7 to 5.9% and between-day from 2.6 to 15.3%. Simplicity and simultaneous detection of ATP and its metabolites make this method suitable for clinical pharmacokinetic studies.

1. Introduction

The nucleotide profile of blood is relatively simple compared to that of nucleated cells. Because no DNA synthesis occurs, only ribonucleotides are present. The adenine ribonucleotide pool, which in metabolically normal erythrocytes mainly consists of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), is much larger than that of other nucleotides, such as the guanine ribonucleotides (GTP, GDP, and GMP)[1]. Generally, ADP and AMP concentrations are only 12.5-20% and 1-2% respectively, of the ATP concentrations[2]. Apart from its role as an intracellular energy carrier, extracellular ATP is involved in processes such as neurotransmission, mechanosensory transduction, secretory functions and vasodilatation, and long-term (chronic) signalling functions in development and regeneration of cells[3]. Moreover, once ATP is released, for example through cell lysis during organ injury, it can mediate several inflammatory responses, including the release of cytokines, tumor necrosis factor-α and interleukin-1β from monocytes and macrophages, and facilitation of leukocyte adhesion to the endothelium[4,5]. Proliferation of various in vitro tumor cell lines has been shown to be suppressed after exposure to ATP[6-8]. The pharmacological use of ATP has received increasing attention following reports of its benefit in pain, vascular disease and cancer[9]. Given our interest in the metabolism of ATP and its behaviour after ATP supplementation in humans, we needed a quick and reliable method for the simultaneous measurement of purine nucleotides, nucleosides, and bases in human blood. Given the complexity of the nucleotide breakdown cascade, which involves various enzymes, special care had to be taken to suppress unwanted nucleotide degeneration in fresh samples.

Several methods have been published to measure nucleotides, including an enzyme assay[10], bioluminescence, 31P nuclear magnetic resonance spectroscopy (NMR), high-performance capillary electrophoresis (HPCE), gas chromatography (GC), ion-pair reversed phase HPLC[11-16], gradient HPLC[17-19], and ion-exchange methods[20]. RP-HPLC lacks some of the drawbacks of other methods, like, for ion-

exchange HPLC, the need for highly concentrated elution buffers and long analysis times. An advantage of the method is that it allows simultaneous detection of nucleotides, nucleosides and nucleobases in a single run with short times between injections. Given the high concentrations of adenine nucleotides in human blood (1500-1900 µmol/L)[21], there was no need for measurement in the lower nanomolar range, which is for instance achievable by bioluminescence kits.

In the present paper we report a reversed-phase HPLC method for the simultaneous analysis of ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, and uric acid in human blood. Sample collection and processing methods were optimized. The application of the method is illustrated by an experiment in which the concentrations of adenine nucleotides are monitored after incubation of human blood samples with ATP, a model used in our lab to study anti-inflammatory properties of ATP and its breakdown products.

2. Materials and methods

2.1. Chemicals

Adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-diphosphate disodium salt (ADP), adenosine 5'-monophosphate sodium salt (AMP), adenine, inosine, hypoxanthine and uric acid were purchased from Sigma Chemical Co., St. Louis, USA. Adenosine was obtained from Bufa B.V., Uitgeest, The Netherlands. Perchloric acid 70% solution in water (PCA) was purchased from Sigma-Aldrich, Steinheim, Germany. Potassium hydroxide (KOH), potassium dihydrogen phosphate (KH_2PO_4), potassium carbonate (K_2CO_3), di-potassium hydrogen phosphate trihydrate ($K_2HPO_4*3H_2O$) and sodium hydroxide (NaOH) were obtained from Merck, Darmstadt, Germany. 0.9% saline was purchased from Braun, Melsungen, Germany. RPMI 1640 (order number 21875) medium containing L-glutamine, was obtained from Gibco, Paisly, UK.

2.2. Sample preparation

Blood was collected from healthy volunteers by venipuncture in EDTA-containing vacutainer tubes (Vacutainer, Becton-Dickinson, New Jersey, USA). 500 µL of blood was vortex-mixed for 5 s with 500 µL of ice-cold 8% PCA in a 1.5 mL Eppendorf tube. After precipitation of the protein fraction (at 12,000 × *g*, 10 min, 4 °C), insoluble perchlorate was formed by addition of 40 µL 2 M K_2CO_3 in 6 M KOH to 650 µL of supernatant, sufficient to neutralize the pH of the samples. Following centrifugal removal of the perchlorate (12,000 × *g*, 10 min, 4 °C), 40 µL supernatant was mixed with 160 µL of mobile phase A (see paragraph 2.4 for its composition) in HPLC microvials (Agilent Technologies, Palo Alto, CA, USA).

2.3. Equipment

ATP and its metabolites were quantified using a series 1100 Agilent HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a quaternary gradient pump, a variable wavelength detector (set at 254 nm) and a solvent degasser system. Separation was achieved with a 3 µm particle size Hypersil ODS C18 RP column (150 × 4.6 mm i.d.; Thermo Electron Corp., USA) with pore diameter 120 Å, protected by a 5µm Hypersil C18 guard column (10 × 4 mm i.d.; Thermo Electron Corp).

2.4. Separation

A 50 mM phosphate buffer (pH 6.0) (mobile phase A), 100% methanol (mobile phase B) and a flow of 1 mL/min were employed to separate the compounds of interest. Buffers were prepared by diluting 50 mL of a stock solution of 0.6 M K_2HPO_4 / 0.4 M KH_2PO_4, to 1 L of milliQ water. The pH was adjusted to 6.0 with concentrated phosphoric acid. From 0-2 min, the eluent consisted of 100% mobile phase A. Between 2-10 min the amount of mobile phase B increased linearly to 12.5%, and then stayed like that for 2 min. Finally, between 12 and 17 min, the gradient returned linearly to 100 % mobile phase A. The column was equilibrated between injections for 2 min, leading to a total run time of 19 min. 25 µl of the sample was injected. The autosampler temperature was set at 4 (± 2) °C. The column was kept at room temperature (20 ± 2 °C).

2.5. Standard solutions

All purine standards were treated in a manner similar to the blood samples. First, the standards were individually dissolved in mobile phase A to a concentration of 4 mM (ATP: 8 mM). Uric acid was dissolved in 0.1 M NaOH instead of mobile phase A. Next, equal volumes of four compounds were mixed and added to 8% PCA in a final ratio of mobile phase A to 8% PCA of 1:1. This resulted in two mixtures with concentrations of 500 µM of each compound (ATP: 1000 µM). The mixtures were subsequently prepared as described in paragraph 2.2 and finally diluted with mobile phase A to acquire a standard range which reflects pre-preparation concentrations of 10 to 500 µM (ATP: 20-1000 µM). Concentrations

of ATP and its metabolites in blood were determined by comparing peak areas to appropriate standards using Chemstation software (Version A.09.03; Agilent, Palo Alto, CA, USA). A mathematical adjustment is applied to correct for the experimentally determined 20% increase in metabolite concentration measured in the supernatant, which occurs due to protein precipitation in blood samples. The stability of ATP was determined using 500 µM ATP solutions in mobile phase A that were a) stored at -20 °C for 2 or 7 days, b) stored at 4 °C for 1 day, or c) incubated at 37 °C for 1 hour. The percentage of degradation products formed was used as a measure for the amount of degradation.

2.6. Assay validation

Linearity of the assay was assessed using three calibration curves analyzed on separate days. The curves were constructed by plotting the peak areas against the concentration of the sample.

The within-day coefficient of variation (CV) was determined by repeated analysis of five aliquots of a single volume of whole blood. The blood was spiked with high and low concentrations of standards. Between-day CV was determined by analyzing, on five different days, a blood sample that was collected from one subject, mixed with PCA and then stored in five aliquots at -20 °C awaiting further sample preparation and analysis. The noise was determined by Chemstation software in the time range between 10.5 and 13 min after injection of ten separate whole blood samples. The limit of detection (LOD) and lower limit of quantification (LLOQ) were calculated by multiplying the SD of the noise by 3 and 10, respectively. The values for all compounds are presented as concentrations in whole blood, in order to present the actual limits that are achievable by our method.

Recovery data of all eight compounds were obtained in duplicate by adding known amounts of nucleotide standards (individually dissolved in mobile phase A (uric acid in 0.1 M NaOH)) or a blank solution (mobile phase A) to blood samples immediately after collection, at high and low spike concentrations. Preparation then continued as described above for both these samples and for samples containing spikes in buffer. Recovery was calculated as: Recovery % = 100 × ([blood + spike)] – [blood + blank]) × ([spike])$^{-1}$.

2.7. Optimization

Sample handling: By using the method of Schweinsberg et al.[2] as a starting point, optimizations were performed with blood samples obtained from healthy volunteers. The optimization of sample preparation included varying a) the anticoagulant in the blood collection tube (EDTA or Li-Heparin), b) the timing of protein precipitation by PCA after blood collection (directly after blood collection or after storage at -80 °C), c) thawing conditions after cold storage (at room temperature or on ice), d) the precipitation of PCA (separate from, or simultaneous with pH neutralisation by KOH), and e) the sample pH (pH range 3 to 6). The effects of changing these parameters were rated optically based on peak shape, yield and compound separation on the HPLC chromatogram.

Chromatographic conditions: The parameters varied for optimization of the chromatographic conditions were a) elution buffer pH (range 3 to 6) and phosphate concentration (range 50- 100 mM), b) timing of the elution gradient, and c) injection volume.

2.8. Application

After optimization, we applied the method to investigate the metabolism of ATP added to whole blood ex vivo, and more specifically, the effects of dilution of the blood with saline or RPMI 1640 medium on the concentration of ATP. This ex-

periment was a follow-up to an earlier study by Swennen et al.[22], in which the ex vivo whole blood model was used to investigate the immunoregulatory effects of ATP. In this model, whole blood was diluted four-fold with RPMI 1640 medium, resulting in an approximate ATP half life of 2 hours. We set out to determine the effect of the dilution factor and type of dilution medium on the ATP metabolism, since literature reports half times of 15 min in undiluted set-ups[23].

Blood from a healthy volunteer was collected in heparin-containing vacutainer tubes and directly put on ice (Vacutainer, 170 IU). Use of EDTA as an anticoagulant was avoided, given our interest in ATP breakdown. Next, appropriate volumes of blood were put in 6- or 24-well plates and spiked with a known volume of 6 mM ATP in milliQ, saline or medium. Next, the spiked blood was used either undiluted or diluted two- or four-fold by mixing gently with saline or medium. The final concentration of ATP was 300 µM in each well. Samples of 450 µL were taken at 0, 2, 4, 8, 15, 30 min and 2, 4, and 24 h after addition of the ATP spike. Plates were incubated at 5% CO_2 and 37 °C between sampling. Upon collection, samples were centrifuged (3000 × g, 10 min, 4 °C), 100 µL of cell-free supernatant was transferred to a clean Eppendorf tube and stored at -80 °C. ATP and its degradation products were determined as described above. Standard solutions were prepared, as described in 2.5., and used to calculate the concentrations of ATP and its degradation products. This resulted in time-dependent degradation profiles for undiluted, and two- and four-fold diluted blood.

3. Results

3.1. Optimization of the sample handling

The goal of optimizing sample handling was to prevent loss of adenosine nucleotides, which are normally rapidly degraded by ecto-enzymes located on the plasma membrane. Lower concentrations of nucleotides were present when blood collection tubes containing the anticoagulant Li-heparin were used instead of EDTA-containing tubes (data not shown). Furthermore, the precipitation of proteins by mixing blood with PCA was found to be most effective in retaining endogenous nucleotides when done directly following blood collection. This in comparison to the situation in which the whole blood was mixed with PCA after it had been stored at -80 °C and then thawed. The ATP concentration was lowered on average by 8% in the latter case. The samples of blood mixed with PCA could either be stored at -80 °C or processed further for direct HPLC measurement without affecting the nucleotide concentration. After cold storage, equal levels of adenine nucleotides were found, regardless of whether the samples were thawed on ice or at room temperature (data not shown).

During sample preparation, it was found that precipitation of PCA in the samples with K_2CO_3 could be performed together with neutralization by KOH without loss of nucleotides. Optimal yield and peak shapes were obtained when all samples were neutralized with buffer to pH 6.0. At pH 3, 4 and 5, peak shapes notably worsened, affecting separation and yield of the various compounds. An example of the effects of buffer pH on the ATP peak is displayed in Figure 1.

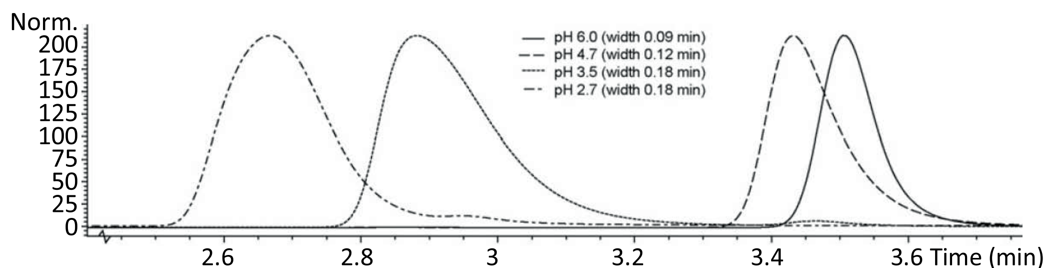

Figure 1: Chromatogram showing the effects of buffer pH on peak shape and retention time after separate injections of ATP (100 µmol/L). Peaks are overlaid on the same time-axis, and normalized to equal height on the *Y*-axis. Only the relevant part of the *X*-axis is shown. Widths reported were taken at half height of the peaks.

3.2. Optimization of chromatographic conditions

Peak shape and compound separation benefited from a buffer pH of 6.0 and a phosphate concentration of 50 mM (data not shown). The elution gradient used was a compromise between a fast analysis time and sufficient separation of the peaks. Higher methanol concentrations resulted in overlapping peaks of the compounds of interest. By returning the methanol concentration to 0 twelve min after injection, the amount of time needed for column equilibration was limited to two min. Finally, the sample injection volume was set at 25 µL, since higher loading volumes resulted in worse separation of ATP and uric acid, particularly in blood, in which both are the most abundant compounds. A representative chromatogram of the separation of eight purine standards in one run is shown in Figure 2.

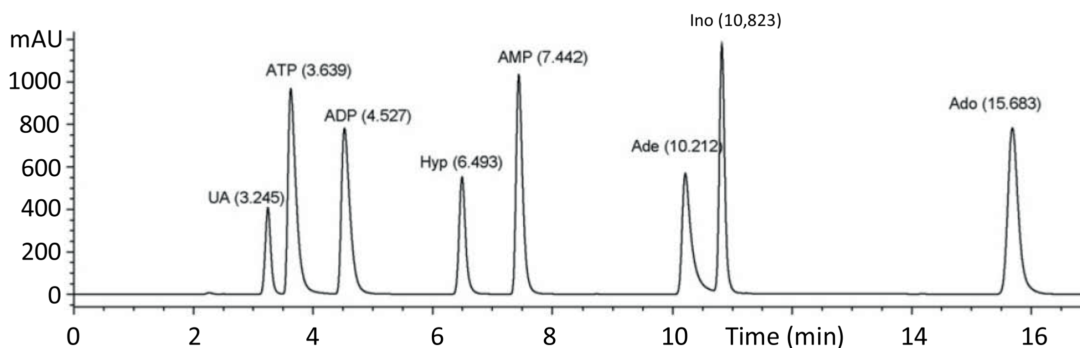

Figure 2: Chromatogram of 500 µmol/L of uric acid (UA), ATP, ADP, hypoxanthine (Hyp), AMP, Adenine (Ade), inosine (Ino) and adenosine (Ado). Conditions of the separation are as described in Section 2.3.

3.3. Assay validation

Chromatograms of unspiked and spiked whole blood samples are shown in Figure 3. Calibration curves were calculated using peak areas at six standard concentrations, the range of which was proportional to the concentration of analyte in the prepared whole blood samples. The concentration range was linear from 1-500 µmol/L for all analytes. The regression coefficient for all calibration curves was greater than 0.999 (Table 1).

Within-day CV (range 0.7 – 5.9%) and between-day CV (2.6 – 15.3%), LOD and LLOQ are presented for every compound in Table 2. The concentrations reported represent the actual LOD and LLOQ of compounds in blood, since dilution of the blood necessary for sample preparation is taken into account. The LOD were all below 0.15 µmol/L and the LLOQ below 0.5 µmol/L.

TABLE 1. SUMMARY OF LINEARITY (RANGE, SLOPE, R2, AND INTERCEPT VALUES) AND ANALYTE RETENTION TIMES. FOR EACH ANALYTE N=9 DATAPOINTS WERE USED.

Analyte	Linearity				Retention time (min)
	Range (µmol/L)	r^2	Slope ± SD	Intercept ± SD	
ATP	1-500	0.999	9.59 ± 0.01	17.27 ± 7.27	3.6
ADP	1-500	0.999	8.25 ± 0.01	8.93 ± 6.62	4.5
AMP	1-500	0.999	8.54 ± 0.01	17.02 ± 6.30	6.5
Hypoxanthine	1-500	0.999	5.55 ± 0.01	42.76 ± 4.36	7.4
Uric acid	1-500	0.999	2.91 ± 0.002	5.77 ± 1.74	3.2
Adenine	1-500	0.999	7.04 ± 0.01	14.35 ± 3.96	10.2
Adenosine	1-500	0.999	10.37 ± 0.02	14.88 ± 8.43	15.7
Inosine	1-500	0.999	8.23 ± 0.07	34.34 ± 2.79	10.8

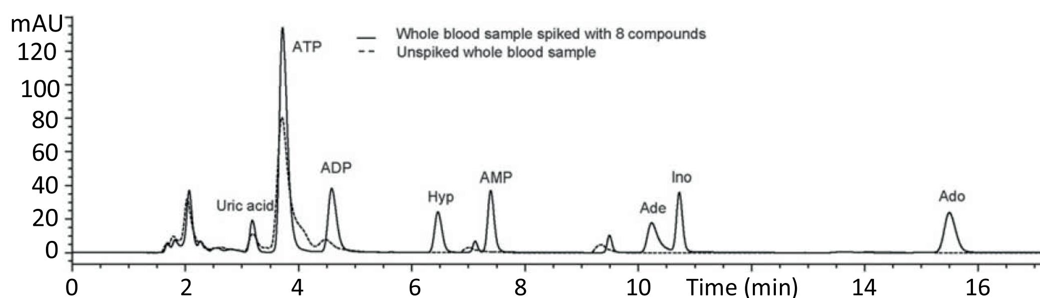

Figure 3: Chromatogram of two whole blood samples. One sample was spiked with a mix of eight compounds (solid line) followed by immediate sample preparation as described in Section 2.2. In the fresh unspiked whole blood sample, only uric acid, ATP, ADP and AMP are detected (dashed line).

The recovery of the method after addition of known amounts of compounds to whole blood samples ranged between 58% and 108% as presented in Table 3. The concentrations of ATP, ADP, and AMP found in the whole blood were 1217 ± 75, 95 ± 15, and 27.1 ± 6 µmol/L, respectively, and the ATP/ADP and ATP/AMP ratios were 12.8 and 44.9, respectively.

The stability of ATP in mobile phase A was assessed for three storage conditions. The proportion of degradation products detected was on average 1% of ATP for storage at 4 °C for 1 day and 37 °C for 1 hour, and 2% for storage and thawing at -20 °C for 7 days.

TABLE 2. SUMMARY OF PRECISION AND DETECTION LIMITS OF ATP, ADP, AMP, HYPOXANTHINE, ADENINE, ADENOSINE, INOSINE AND URIC ACID (CV = COEFFICIENT OF VARIATION, LOD = LIMIT OF DETECTION, LLOQ = LOWER LIMIT OF QUANTIFICATION)

Analyte	Within-day precision		Between-day precision		LOD (µmol/L)	LLOQ (µmol/L)
	Mean (µmol/L), *N*=5	CV (%)	Mean (µmol/L), *N*=5	CV (%)	*N*=10	*N*=10
ATP	1003.0	0.7	974.3	3.6	0.049	0.162
ADP	356.5	0.9	357.3	5.5	0.072	0.239
AMP	241.1	1.3	244.5	2.6	0.052	0.172
Hypoxanthine	169.9	5.9	206.1	15.3	0.082	0.272
Uric acid	304.9	1.6	326.4	11.5	0.121	0.405
Adenine	167.5	3.2	194.4	10.4	0.090	0.299
Adenosine	226.1	1.7	221.8	4.3	0.070	0.232
Inosine	206.0	2.1	234.8	15.0	0.059	0.197

Between-day precision of five aliquots of a single blood sample, measured on 5 different days spread over a period of 7 months. Within-day precision is calculated after repeated analysis *(N*=5) of multiple aliquots of a single volume of whole blood, spiked with known amounts of standards. Values are the mean concentrations and the coefficient of variation (CV). The LOD and LLOQ were calculated as 3 and 10 times the SD of the noise of the UV baseline in untreated whole blood samples (*N*=10) in the time range of 10.5 -13 minutes after injection.

3.4 Application

In this experiment, the effects of dilution of blood with saline or RPMI 1640 medium on ATP metabolism were investigated in an ex vivo set-up. Figure 4 displays the degradation of ATP added at t=0 to the undiluted (4a), and two-fold (4b and 4c) and four-fold (4d and 4e) diluted aliquots of blood. Adenine, adenosine and inosine were not detected. Besides uric acid, none of the other compounds were present in samples taken before the addition of ATP.

In the undiluted situation, the ATP concentration declined steadily until complete degradation after 30 min. The ADP concentration doubled 15 min after incubation, followed by a decline to zero after two hours. AMP rose between 2 and 30 min, with the strongest increase between 15 and 30 min, followed by a decline to zero after four hours. Hypoxanthine was first formed after 30 min incubation and kept rising sharply until the final measurement after 19 hours. In contrast to the other metabolites, uric acid levels were quite stable with only a minor increase at the later stages of the incubation.

In the two-fold diluted situation, every compound behaved similarly, but with a time-frame that was shifted by approximately 90 min. Two notable differences

are a) the near simultaneous disappearance of ADP and ATP, instead of ATP preceding ADP, and b) the smaller rise in hypoxanthine concentration after 19 hours.

TABLE 3. RECOVERY DATA FOR ATP, ADP, AMP, HYPOXANTHINE, ADENINE, ADENOSINE, INOSINE AND URIC ACID IN WHOLE BLOOD

Recovery	Sample conc µmol/L	Added conc µmol/L	New Conc µmol/L	%
ATP	844.0	1000	2057.8	**121.4%**
		500	1422.6	**115.7%**
ADP	124.2	500	749.4	**125.0%**
		250	445.4	**128.4%**
AMP	36.3	500	548.9	**102.5%**
		250	299.5	**105.2%**
Adenine	0	500	540.7	**108.1%**
		250	272.3	**108.9%**
Adenosine	1.53	500	548.4	**109.4%**
		250	275.5	**109.6%**
Inosine	0	500	562.6	**112.4%**
		250	271.1	**108.4%**
Hypoxanthine	3.87	500	481.0	**95.4%**
		250	287.9	**113.6%**
Uric acid	254.6	500	682.1	**85.5%**
		250	465.2	**84.2%**

Known concentrations of standard solution were added (Added conc) to a subject's whole blood sample with predetermined metabolite levels (Sample conc). The resulting metabolite concentration of the mixture (New conc) was determined. The concentrations presented are averages of n=5 untreated blood samples and n=5 mixtures.

Compared to the undiluted situation, the shift in time in the four-fold diluted situation was approximately 3.5 hours. One difference is that the ATP concentration does not decline directly after the start of the incubation, but only after 15 min (Fig. 4d) or 4 min (Fig. 4e). Finally, the increase in hypoxanthine after 19 hours was even smaller, compared to the twofold diluted situation.

No remarkable differences between medium and saline were found. In the four-fold diluted situation, complete degradation of ADP took slightly longer in saline than in medium. Finally, as remarked in the previous paragraph, ATP seems to start degrading somewhat later when diluted in saline rather than medium.

4. Discussion

In the present paper we have optimized and applied a simple and rapid reversed-phase HPLC method for the simultaneous analysis of ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, and uric acid in human whole blood. Crucial steps in the collection, handling and preparation of the blood samples

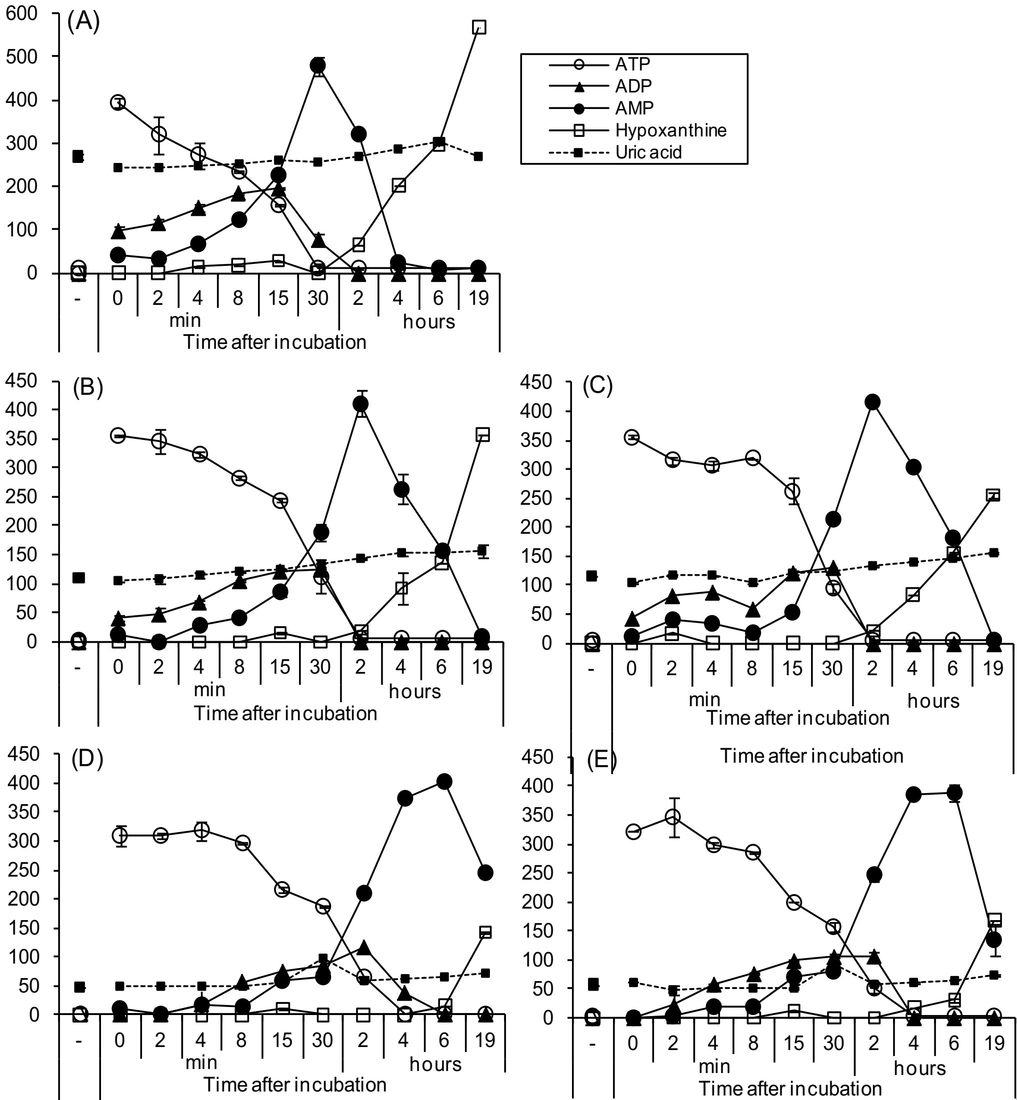

Figure 4: Degradation profile of ATP added to blood that was (A) undiluted, (B) diluted two times with saline, (C) diluted two times with medium, (D) diluted four times with saline, or (E) diluted four times with medium. *X*-axis (not to scale) plots the time in minutes and hours after incubation at t = 0. Concentrations in the samples taken before addition of the ATP spike are represented by —. The legend shown in A applies to all conditions. The concentration (µM) of each of the detected compounds is plotted on the *Y*-axis. Curves represent means and error bars represent standard deviation (n = 2).

include collection in EDTA-tubes, immediate PCA addition, and neutralization before HPLC analysis at pH 6. The faster ATP breakdown in heparin-containing tubes, compared to EDTA-tubes, can be explained by EDTA's Ca^{2+}-chelating activities that inactivate the ATP-degrading enzymes[(24)]. Addition of PCA serves the same purpose, since PCA precipitates all proteins and thus inactivates enzymes as well.

For sufficient inactivation of the enzymes, PCA had to be thoroughly mixed with the blood before freezing the samples. The enzymes were properly inactivated, as shown by the observation that no significant loss of ATP occurred when samples were thawed on ice or at room temperature. Further preparation was not necessary, thus making this method suitable for clinical applications (bedside). Chromatographic separation can be difficult for compounds with multiple pK_a's. Neutralisation of the samples with KOH was necessary before HPLC analysis, and eluent pH values below six resulted in worse shape and separation of the peaks. Finally, the stability of the samples and the standard compounds was such that large batches of samples could be analyzed in one run, without risking degradation.

The recovery levels ranged between 82 and 108% for ATP, ADP, AMP, adenosine and inosine, but were below 70% for uric acid, adenine and hypoxanthine. These low recovery levels may be caused by coprecipitation of these metabolites together with proteins during the centrifugation step of the acid extraction preparation of the samples or with the perchlorate precipitate after neutralization(25,26). In whole blood, precipitate volumes are large, and the 20% adjustment of concentration to correct for this volume change may have been an overcorrection for more apolar metabolites, such as uric acid, adenine and hypoxanthine, resulting in lower recoveries. Recovery levels reported by others for methods using perchloric acid for the extraction of nucleotides from cells or tissues range between 75% and 120.5%(27-30). The recoveries did not differ substantially whether the nucleotides were spiked at high or low concentrations. The whole blood concentrations of ATP, ADP, AMP and uric acid reported in table 3 correlate well with those reported by others(2,31).

The LOD (below 0.15 µmol/L for all compounds) and LLOQ (all below 0.5 µmol/L) were more than adequate for the purpose for which this method was developed: the quantification of ATP and its metabolites in whole blood. Concentrations of ATP in whole blood are extremely high compared to plasma concentrations, because ATP present at millimolar concentrations inside the erythrocytes is released before measuring whole blood. Erythrocyte ATP concentrations exceed plasma ATP concentrations, which are in the nanomolar range, by over ten-thousand-fold(32). A frequently used method to measure ATP is the luciferin-luciferase assay(33,34) This method has the sensitivity needed for the detection of low plasma ATP levels. A recent similar method is presented by Farthing et al.(35). For measurement of ATP in whole blood, extreme dilutions are necessary. However, its main disadvantage compared to our method is that only ATP can be determined, whereas we can quantify the complete adenylate pool in one run. Ion exchange methods often require more extensive pretreatment of samples and have difficulties in separating nucleobases, nucleosides and nucleotides in one run, given the charged nature of the nucleotides in the operating pH range (pH 2-7)(33,36-39).

Adenosine and inosine concentrations in whole blood were below the LOD. This is in line with results of studies investigating the adenine nucleotide content in human whole blood and erythrocytes(40,41). Our within-day CV (range 0.7 – 5.9%)

and between-day CV (2.6 – 15.3%) correlate well with the findings of others[42]. In the application of the method, we found that ATP added to whole blood ex vivo was converted into several intermediate products, which, together, created a typical degradation profile. The 30 min degradation period of ATP in the undiluted sample is consistent with the literature data[23,43]. In a similar set-up, Heptinstall et al.[23] found that the degradation of ATP to AMP in plasma can be attributed mainly to leukocytes, since these possess a high ectonucleotidase activity. When comparing the whole blood model to the in vivo situation, the main difference is the absence of vascular endothelial cells in the model. The ectonucleotidases that are active on the luminal surface of endothelial cells, shorten the ATP half-life to seconds or less in vivo[44]. In an ex vivo set-up, but with endothelial factors present, the half-life of ATP is longer (5-10 min), probably due to the static nature of this setup, in comparison to the in vivo situation in microvasculature[45-47].

In addition to ATP, the degradation profiles of the other compounds also showed similarities to those reported before in the literature[23], even though we employed a longer incubation period and started at a higher ATP concentration. First, as was also reported by Coade and Pearson[45], this profile indicates the sequential catabolism of ATP to ADP and AMP. ADP formation coincided with ATP degradation, whereas the formation of AMP began four min later. The latter indicates that ADP, while still being formed as a degradation product from its precursor ATP, is simultaneously being degraded into AMP. This also becomes clear from the observation that, starting 30 min after incubation, the decline in ADP concentration coincides with the complete depletion of its precursor ATP. The combination of both processes leads to a strong rise in AMP concentration between 30 min and two hours after incubation. Second, the profile shows a strong increase in hypoxanthine concentrations between two and nineteen hours, after which the experiment was terminated. The catabolism of adenine nucleotides beyond AMP has been shown to involve the enzyme 5'-nucleotidase (CD73) to yield inosine monophosphate (IMP), inosine and hypoxanthine[48]. Heptinstall et al.[23,45,49] reported that this enzyme acts independently from leukocytes or erythrocytes, since breakdown of AMP added to cell-free plasma was similar to that in whole blood. In line with other studies[23], no other intermediate products (adenosine and inosine), were detected. Studies with the uptake inhibitor dipyridamole revealed that adenosine is taken up efficiently by the erythrocytes through equilibrative nucleoside transporters after which rapid sequential conversion into hypoxanthine occurs intracellularly, with hypoxanthine finally being distributed outside of the cell, resulting in the observed increased hypoxanthine levels[23,50].

In our experiment, hypoxanthine was the final degradation product and we observed only a very small rise in the concentration of uric acid, which is the endproduct in vivo[51]. This could be explained by the probable absence of xanthine oxidase (EC 1.2.3.2) in our model, the enzyme responsible for the further degradation of hypoxanthine to xanthine and finally uric acid. The highest activity of xanthine oxidase is reportedly found in the liver and intestinal mucosa[52,53], but it is

also present in various organs and vascular endothelial cells[54,55]. Both liver and endothelial cells are absent in our set-up, which explains the lack of change in uric acid levels.

Besides interest in the breakdown profile of ATP added to whole blood, we were also interested in the effects of dilution of the whole blood with two different media: either saline of RPMI 1640 medium. The RPMI 1640 medium is used by Swennen et al.[22] in the whole blood model to more closely resemble the in vivo situation. We therefore hypothesized that addition of this medium would help stabilize ATP more than would a similar dilution with saline. However, degradation profiles in blood diluted with either saline or medium were quite similar. This result indicates that the medium does not contain any substance that might delay the degradation of ATP.

In contrast, the extent of dilution does influence the degradation profile of ATP added to the blood. First, the delay in ATP breakdown got more pronounced with increasing dilution of the whole blood (Fig. 4). For instance, whereas in undiluted blood, ATP completely degraded in 30 min, this process can take up to two and four hours in two-fold and four-fold diluted blood, respectively. Second, other features of the degradation profile were delayed as well, such as the decline in ADP combined with the sharp rise in AMP that coincides with ATP depletion at 2 hours. Furthermore, the maximum level of AMP was reached after incubation of two hours (two-fold diluted) or after incubation of four to six hours (four-fold diluted).

5. Conclusion

The HPLC procedure described in this paper allows separation of the main metabolites of adenine nucleotide metabolism found in human whole blood samples. Compared to previous methods, it is a simple and rapid procedure, which has a short run time, good peak shape and high sensitivity. The method has been validated with respect to accuracy, precision, linearity and limit of detection, recovery and stability. The method is suitable for purine analysis in vivo or in an ex vivo set-up, enabling nucleotide metabolic processes to be followed in time. It has been reported for long that lowered blood ATP levels have been associated with acute disease states. Decreased levels of adenine nucleotides in erythrocytes were, for instance, observed in patients with various malignancies[56,57]. The method described here may be used to monitor changes that occur in these patients and after therapeutic administration of ATP.

Acknowledgements

The authors thank B. Vriens, M. de Vaan, C. van den Hurk, H. Bos and A. Skrabanja for their contributions to the method development and M. Fischer and R. Bartholomé for their excellent technical assistance.

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Chapter 3

Oral supplements of adenosine 5'-triphosphate (ATP) are not bioavailable: a randomized, placebo-controlled cross-over study in healthy humans

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Abstract

ATP is present in substantial concentrations in certain foods and in breast milk, it is available as an orally administered drug used for analgesic purposes, and it is being marketed as a sports aid. Several studies have reported beneficial effects of oral ATP administration, even though its bioavailability is unclear. We investigated whether targeted delivery of ATP to the small intestine by using enteric-coated pellets would lead to an increase in whole blood concentrations of ATP and/or its metabolites.

Methods: Eight healthy volunteers participated in a cross-over study. They were given in random order single doses of 5000 mg ATP or placebo via two types of pH-sensitive, enteric-coated pellets (targeted at release in the proximal or distal small intestine), or via a naso-duodenal tube. Blood ATP and metabolite concentrations were monitored by HPLC for 4.5h (duodenal tube) or 7h (pellets) post-administration. Areas under the concentration vs. time curve were calculated and compared by paired-samples t-tests.

Results: Except for uric acid, no significant changes in ATP and metabolite concentrations were observed. Significantly increased uric acid concentrations of approximately 50% were found for proximal-release pellets (P=0.003) and for duodenal tube (P=0.001) compared to placebo, whereas distal-release pellets failed to increase uric acid concentrations.

Conclusion: A single dose of orally administered ATP is not bio available as such. However, similar increases in its final metabolite uric acid are found when ATP is administered by proximal-release enteric coated pellets or duodenal tube. Uric acid itself may have beneficial health effects. Further studies will be necessary to determine whether prolonged daily administration of ATP will enhance its oral bioavailability.

Introduction

The purine nucleotide adenosine 5'-triphosphate (ATP) is found in all cells of the human body, where it functions as a source of energy and as a co-factor in cellular metabolism. ATP can also be released from cells to act as a local regulator of neurotransmission, secretory functions, inflammation, and nociception via interaction with purinergic receptors[1, 2]. ATP is present in substantial concentrations in a number of foods (e.g. meat, soy, mushrooms)[3] and in breast milk[4, 5]. Furthermore, capsules containing ATP are currently registered in France for the treatment of low back pain of muscular origin, and supplements containing ATP are marketed on the internet for various purposes including the restoration of energy.

Several studies have found potentially beneficial effects of oral ATP administration. In an experimental study by Jordan *et al.*[6], three groups of nine healthy men received ATP (150 or 225 mg) or placebo for 14 days. Physical performance

and muscular strength were positively affected, although no significant differences were observed in whole blood and plasma ATP concentrations between the ATP and placebo groups. Another study investigated the effects of 30 day supplementation with an ATP-containing registered drug (Atépadène®, 90 mg daily)[7, 8]. The questionnaire-based outcome indicated that it provided some benefit to patients with subacute low back pain. The positive results of ATP in the study by Jordan *et al.*, investigating muscular power, have also promoted the commercial marketing of ATP as an aid in sports, especially in the area of bodybuilding[6]. Animal studies reported alterations in cardiac, vascular and pulmonary function after 30 days of oral ATP supplementation[9, 10]. Whereas, like in humans[6], no changes in plasma ATP or metabolites were detected in the systemic circulation, the concentrations of adenine, inosine, adenosine, adenosine monophosphate (AMP) and uric acid in plasma from the portal vein were increased. The authors concluded that these purine nucleosides can be absorbed from the intestinal lumen and secreted into the portal bloodstream. The identification of a number of nucleoside transporters in the small intestine further suggested that orally administered ATP can be absorbed and may be utilized by the human body[11].

We have previously shown that intravenous administration of ATP in several randomized controlled trials prevented weight loss, fatigue, lowering of serum albumin concentrations, deterioration of muscle strength, and improved functional performance (walking stairs, household activities etc.) and overall quality of life in lung cancer patients[12, 13]. Suggested mechanisms of action included repletion of intracellular energy stores[14] and inhibition of the acute phase response[15-17]. Although successful, the clear drawbacks of intravenous infusions prompted us to explore the possibility of an oral route of administration of ATP.

We hypothesized that targeted delivery of ATP to the small intestine using enteric coated pH-sensitive multi-particulate formulations (pellets) will lead to an increase in whole blood concentrations of ATP and/or its metabolites. We further hypothesized that oral administration with pellets is as effective as direct administration of ATP into the duodenum using a naso-duodenal tube. Finally, we investigated whether absorption of ATP differs between distinct regions of the small intestine.

Materials and methods

Study design

Subjects were examined on five occasions in a cross-over design. On days 0, 7 and 14, subjects received the following ATP doses in random order: 5000 mg ATP as proximal-release or distal-release pellets, or placebo proximal-release pellets. The pellets were ingested with approximately 200 ml water acidified to $pH < 5$ with citric acid. On days 21 and 28, subjects received in random order 5000 mg ATP dissolved in 100 ml water (30 ± 4°C), or water only (placebo), administered

through a naso-intestinal tube. The tube was inserted through the subjects' nostril and placed in the stomach. To promote movement of the tube through the pylorus into the duodenum, subjects were asked to lay down on their right side. To verify the tube's position (either stomach or duodenum), gastro-intestinal juice samples were taken by a syringe and tested for their pH and color. Once pH was above 5 (±180 min after insertion of the tube), and color was yellow, administration started and the tube was removed 10 min later.

Study population

Male and female subjects (18-60 years) received oral and written information about the protocol and possible risks before signing informed consent. Exclusion criteria were a history of lung, heart, intestinal, stomach or liver disease, use of prescription medication, smoking, drug use, dietary restrictions, and pregnancy. Subjects abstained from products containing alcohol or caffeine and from purine-rich foods, such as game, offal, sardines, anchovies and alcohol-free beer for two days before each test day. Subjects fasted from 10 p.m. the previous day until the end of the test day (4 p.m.), and refrained from any vigorous physical activity 24 h before each test. Subjects were allowed to drink water starting 30 min after ATP or placebo administration. The study was approved by the Medical Ethics Committee of Maastricht University Medical Centre. The study was carried out according to the Helsinki Declaration for human experiments.

Materials

ATP disodium salt was purchased from Pharma Waldhof GmbH, Düsseldorf, Germany. Adenosine diphosphate (ADP) disodium salt, AMP sodium salt, adenine, inosine, hypoxanthine, uric acid and nitric acid were purchased from Sigma Chemical Co., St. Louis, USA. Adenosine and lithium carbonate (Li_2CO_3) were obtained from Fagron BV., Uitgeest, The Netherlands. Perchloric acid (PCA) 70% solution in water was purchased from Sigma-Aldrich, Steinheim, Germany. KOH, KH_2PO_4, K_2CO_3, K_2HPO_3*$3H_2O$ and NaOH were obtained from Merck, Darmstadt, Germany and 0.9% saline from Braun, Melsungen, Germany. Bengmark-type naso-duodenal tubes were from Flocare, Zoetermeer, The Netherlands. NH_4NO_3 was obtained from Fluka, Steinheim, Germany. Trichloroacetic acid (TCA) 20% solution in water was from Serva, Heidelberg, Germany. Citric acid suitable for human consumption was obtained from the pharmacy of Maastricht University Medical Centre.

Production of pellets

ATP pellets were produced at Ghent University, Faculty of Pharmaceutical Science, Belgium as described by Huyghebaert et al.[18], with minor modifications to obtain an ATP concentration of >40% (wt:wt) after coating. Placebo pellets were produced similarly, but without ATP. To investigate the timing of intestinal release, Li_2CO_3 (60 mg per administration) was added to the pellets.

The proximal-release pellets were coated with 30% Eudragit® L30D-55 (ATP or placebo pellets), and the distal-release pellets (ATP only) were coated with 15%

Eudragit® FS 30 D (Röhm Pharma, Darmstadt, Germany), mixed with anionic copolymers of methacrylic acid and ethylacrylate (1:1). After coating, the pellets were cured overnight at room temperature at 60% (proximal-release pellets) or 20% (distal-release pellets) humidity, packed in aluminium foil sachets (VaporFlex®, LPS, NJ, USA), sealed at their respective humidity and stored at room temperature. Pellets were used within 3 months after production.

Dissolution testing

To test whether the coating of the pellets was adequate, a dissolution test (n = 3 for each type of coating) was performed using the reciprocating cylinder method (USP apparatus 3 from Bio-Dis, VanKel, NJ, USA) at a dip rate of 21 dips per minute using 3 g pellets per vessel (250 ml) with two consecutive media: 0.1 N HCl (37°C), and a 0.2 M KH_2PO_4 buffer (37°C) with a pH that was adjusted to 6.5 for the proximal-release pellets, and pH 7.4 for the distal-release pellets. Samples were collected after 2 h in HCl and after 2, 5, 10, 20, 30 and 60 min in buffer as described in Huyghebaert et al.[(18)]. ATP and metabolite concentrations were measured by HPLC separation and UV-analysis as previously described[(19)].

Sample collection during the intervention

Venous blood was collected from the antecubital vein by a 20 gauge intravenous catheter (Terumo-Europe NV, Leuven, Belgium), connected to a three-way stopcock (Discofix®, Braun Melsungen AG, Melsungen, Germany). Blood was collected into 4 ml EDTA tubes (Venosafe, Terumo-Europe NV) by inserting a 21 gauge multisample needle (Venoject Quick Fit, Terumo-Europe NV) into the membrane of a closing cone (IN-Stopper, Braun Melsungen AG) that was attached directly to the stopcock. The anticoagulant EDTA inhibits the extracellular hydrolysis of ATP by Ca^{2+}- and Mg^{2+}-activated enzymes, like plasma membrane-bound CD39[(20)]. To avoid clotting after each blood collection, approximately 1.5 ml of heparinised (50 I.E./ml) 0.9% saline was used to rinse the blood collection set-up. It was removed before the next blood collection.

Three baseline blood samples were collected at 30, 20 and 10 min before administration. Starting 30 min after pellet administration or 15 min after duodenal administration, blood samples were collected every 15 min. Between 210 and 420 min (pellets) or 270 min (duodenal tube) after administration, samples were collected every 30 min. Total volume collected per day was 92 ml.

After blood collection, the tubes were inverted three times and put on ice. Five hundred µl of blood was added to 500 µl ice-cold PCA (8% wt:v), vortex-mixed and frozen in liquid nitrogen. To determine the concentration of ATP and all metabolites except uric acid in the erythrocyte fraction, while measuring whole blood samples, the measured concentrations were divided by the hematocrit (determined using a microhematocrit method[(21)]). Untreated plasma samples (centrifugation at 3000 rpm, 10 min, 4°C) were collected for assessment of lithium release from the pellets. All samples were stored at -80°C awaiting analysis.

ATP measurement in whole blood samples by HPLC

Equipment, sample preparation and measurement conditions have been previously described and validated[19]. Briefly, after thawing, the protein fraction was precipitated (12,000 X g, 10 min, 4°C) and 40 µl 2 M K_2CO_3 in 6 M KOH was added to 650 µl supernatant to neutralize the pH. The resulting insoluble perchlorate was removed by centrifugation (12,000 X g, 10 min, 4°C), and 40 µl supernatant was mixed with 160 µl 0.05 M phosphate buffer pH 6.0 in HPLC vials.

Lithium measurement in plasma samples

To investigate the timing of pellet disintegration, plasma concentrations of the lithium marker were examined in plasma using a modified Trapp protocol[22]. Following thawing on ice, 50 µl plasma was vortex-mixed with 10 µl trichloroacetic acid (20% v:v) and centrifuged (14,000 rpm, 10 min) to precipitate the proteins. The supernatant was diluted 20x in 0.1 M nitric acid, which also served as the blank. Two replicate measurements per sample were performed on a SpectrAA 400 graphite tube atomic absorption spectrophotometer (AAS) (Varian, Palo Alto, CA, USA) with a lithium hollow-cathode lamp, operated at 5 mA and a 1.0 nm slit. Peak height measurements at 670.8 nm wavelength were compared with values for standards of known concentrations (ranging from 2-10 ng/ml). Initially 20 µl sample and 5 µl modifier solution (1.2 M NH_4NO_3) were injected into the top hole of the graphite tube. Then, fluids were evaporated at 95°C for 40 s and at 120°C for 10 s. The ash time was 15 s at 700°C, followed by atomization at 2300°C with a 3 s read time. If the obtained signal exceeded the standard concentration range (0-10 ng/ml), samples were diluted with blank and measured again.

To evaluate lithium content of the pellets before use, uncoated placebo-pellets were mixed thoroughly in buffer and samples were taken at 10, 20, 30 and 60 min after dissolution. These samples were analysed for lithium content using the above protocol.

Statistical analysis

The area under the concentration vs. time curve (AUC) was calculated using the linear trapezoidal rule from time zero until the last time point of sampling *t* (AUC_{0-t}). C_{min} and C_{max} were defined as the minimum and maximum observed concentrations, respectively. t_{max} was the time of C_{max}. AUC of the five conditions were compared and analyzed by paired-samples t-tests. $P<0.05$ was considered statistically significant.

Results

Study population

Eight subjects (6 females, 2 males aged 26.9 ± 5.9 (mean ± SD), weighing 70 ± 4.3 kg, with a BMI of 23.6 ± 1.3) completed the trial. No adverse events were observed with both modes of administration (i.e. pellets, solution).

Dissolution testing

First, the lithium content of the uncoated pellets was verified in vitro by AAS to be 0.6% (wt:wt) (data not shown). Next, the coating properties and ATP-yield of the pellets were tested in a dissolution experiment lasting 180 min. **Fig. 1** shows the percentage of ATP that was released, with 100% representing the concentration of the sum of ATP plus its metabolites at 180 min. During 120 min in 0.1 N HCl, less than 5% ATP and metabolite (5.0 ± 0.6% for the proximal-release pellets and 3.4 ± 0.4% for the distal-release pellets) was released. Subsequent rapid changing of the buffer solutions to pH 6.5 or 7.4 for 60 min caused a release of 50% of the remaining ATP within 5 min (proximal-release pellets) or 25 min (distal-release pellets), which increased to >80% after sixty min. ATP was partially broken down to ADP (8.6% for proximal-release pellets and 7.0% for distal-release pellets), AMP (1.0 and 0.7%, respectively), and uric acid (4.0 and 2.5%, respectively).

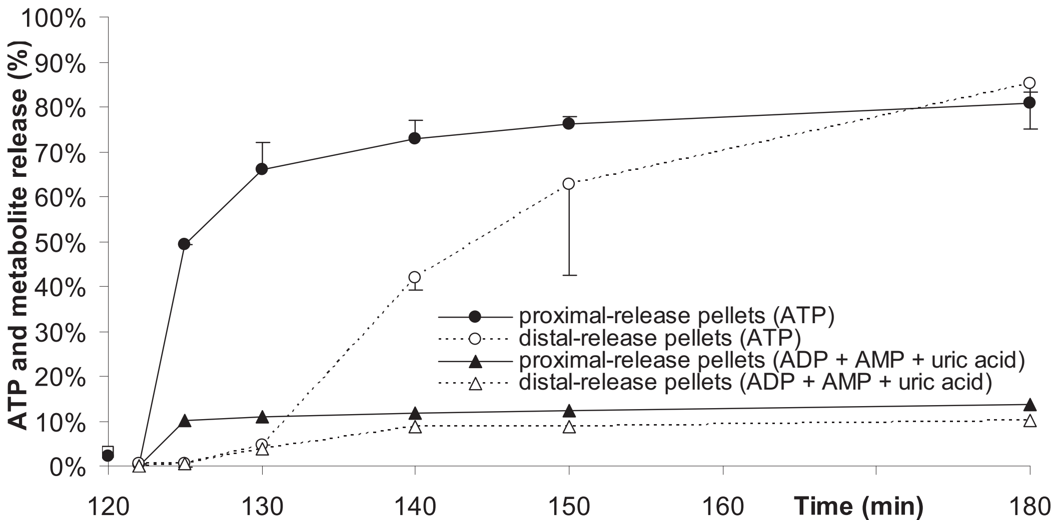

Figure 1: Release profiles of ATP and metabolites from proximal-release (closed symbols) and distal-release (open symbols) pellets after 120 min in 0.1 N HCl (time axis has been shortened between 0 and 120 min) and subsequently 60 min in buffer solutions with either pH 6.5 (proximal-release pellets) or 7.4 (distal-release pellets). The Y-axis represents the percentage of the total amount of ATP and metabolites released after 60 min. Data were obtained by the reciprocating cylinder method (USP apparatus 3). Values are means ± SEM, *n* = 3

Lithium in plasma

Fig. 2 depicts mean plasma lithium concentrations in samples collected for 7 h after administration of the coated pellets. The three types of pellets had different release profiles, as was quantified by measuring the AUC (**table I**). Comparison of the AUC of the ATP-containing pellets revealed that the proximal-release pellets caused a significantly higher increase in plasma lithium than the distal-release pellets ($P = 0.001$) (Fig. 2). Further comparison of the proximal-release pellets with or without ATP, showed that the lithium AUC was significantly lower in the ATP-containing pellets than in the placebo-containing ones ($P = 0.001$). Individual plasma lithium concentrations are depicted in **Supplemental fig. 1** (after reference section). Lithium C_{max} for the proximal release pellets was reached between 135 and 210 min after administration at a mean concentration of 404 ng/ml for the placebo pellets and 200 ng/ml for the ATP pellets. The highest plasma lithium concentration (717 ng/ml) was measured in a volunteer receiving placebo proximal-release pellets. The distal-release pellets, on the other hand, showed a delayed and

TABLE 1. EFFECT OF ATP VS. PLACEBO ADMINISTRATION ON URIC ACID AND LITHIUM CONCENTRATIONS

Formulation	*Δ AUC uric acid mmol.min/L*	*C_{max} mmol/L (range)*	*t_{max} min (range)*	*AUC Lithium mmol.min*
Duodenal tube[g]				
ATP 270 min	19.6 ± 4.4 [a,b,c]	0.31 ± 0.03 (0.23-0.38)	135 (105-240)	n.a.
Placebo 270 min	-0.4 ± 0.4	0.21 ± 0.03 (0.15-0.33)	n.a.	n.a.
Proximal release pellets[h]				
ATP 270 min	16.1 ± 3.0	n.a.	n.a.	n.a.
Placebo 270 min	0.8 ± 0.9	n.a.	n.a.	n.a.
ATP 420 min	25.4 ± 5.7[d,e]	0.30 ± 0.03 (0.21-0.41)	240 (165-390)	65174 ± 7985[f]
Placebo 420 min	0.9 ± 1.1	0.20 ± 0.02 (0.16-0.31)	n.a.	117914 ± 15021[f]
Distal release pellets[i]				
ATP 270 min	1.7 ± 1.1	n.a.	n.a.	n.a.
ATP 420 min	3.2 ± 1.4	0.22 ± 0.02 (0.17-0.34)	390 (105-420)	12575 ± 2832[f]

Values are group means ± SEM, *n* = 8 per formulation, paired-samples t-tests.
[a] Different from duodenal tube placebo (*P*=0.002), [b] Different from ATP distal-release pellets 270 min (*P*=0.007), [c] Different from proximal-release placebo pellets 270 min (*P*=0.007) [d] Different from ATP distal release pellets 420 min (*P*=0.005), [e] Different from proximal-release placebo pellets (*P*=0.005), [f] Different from each other (*P*<0.001) [g] 5000 mg ATP dissolved in 100 ml water or placebo was administered to healthy volunteers using a naso-duodenal tube. [h] 5000 mg ATP or placebo was administered to healthy volunteers by means of an enteric coated pH-sensitive multi-particulate formulation that will release its content once pH is above 6.5. [i] 5000 mg ATP or placebo was administered to healthy volunteers by means of an enteric coated pH-sensitive multi-particulate formulation that will release its content once pH is above 7.4.

lower release profile, with lithium concentrations starting to rise only approximately 240 min after administration, while a maximum concentration of 103 ng/ml was reached at the final measurement.

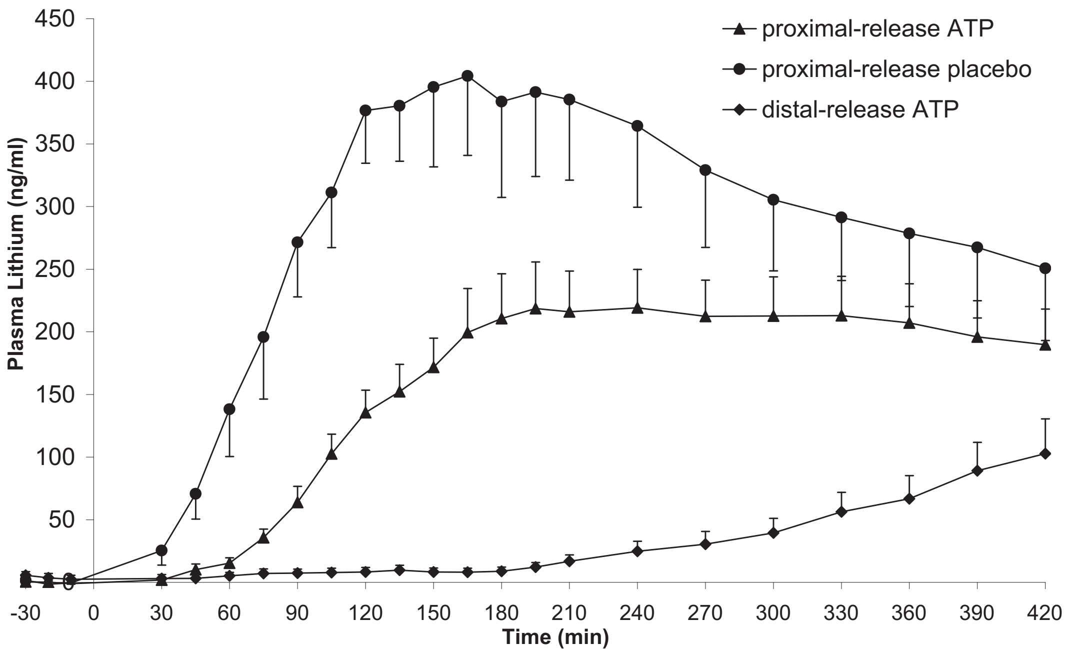

Figure 2: Plasma lithium concentrations after administration of a dose of pellets containing 60 mg Li_2CO_3 to healthy volunteers. Following ingestion of the proximal-release pellets without ATP (-●-), the plasma lithium concentration rose earlier and higher compared to pellets with the same coating but with ATP (-▲-). Following ingestion of proximal-release pellets, higher plasma lithium concentrations were attained compared to distal-release pellets (-◆-). Significant differences between the pellets are based on the AUC and can be found in table I. Values are means ± SEM, *n* = 8.

ATP and metabolites in blood

HPLC analysis of the whole blood ATP concentration revealed no statistically significant differences between placebo and ATP for any type of administration (data not shown). In fact, of the other metabolites (ADP, AMP, adenosine, adenine, inosine, hypoxanthine and uric acid), only uric acid concentrations changed (**Fig. 3**). Compared to placebo, the uric acid AUC increased significantly when ATP was administered by proximal-release pellets (P = 0.003) or by duodenal tube (P = 0.001). Administration of ATP by distal-release pellets did not lead to a significantly increased uric acid AUC, compared to placebo. The peak uric acid concentrations (C_{max}) were 36% higher (0.28 ± 0.02 mmol/L) for proximal-release pellets compared to distal-release pellets (0.21 ± 0.01 mmol/L), but 6% lower compared to the administration via duodenal tube (0.30 ± 0.02 mmol/L) (Fig. 3 and statistics in table I). The mean time to peak uric acid concentration (t_{max}) was shorter for duodenal tube

administration (t_{max} ranged from 75 to 195 min with mean ± SD 135 ± 15 min) as compared to the pellet administration (t_{max} ranged from 150 to 390 min with mean ± SD 234 ± 32 min). An overview of the inter-subject variability in uric acid concentrations following administration of ATP (tube and pellets) is presented in **Supplemental Figure 2** (after references).

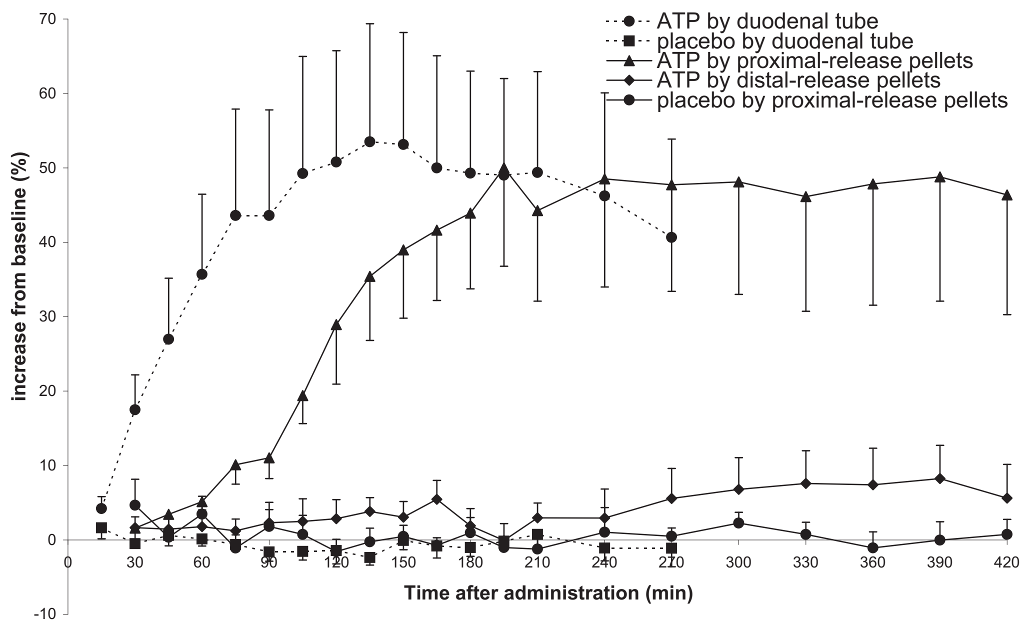

Figure 3: Uric acid concentrations in healthy volunteers supplemented at t=0 with a single dose of 5000 mg ATP or placebo. Data are presented as percentages increase from the mean of three blood samples taken before administration. Significant differences between the administrations are based on the AUC and can be found in Table I. Values are means ± SEM, *n* = 8

Discussion

The aim of this study was to investigate whether targeted delivery of ATP to the small intestine using two types of enteric coated pH-senstive multi-particulate formulations (pellets) would lead to an increase in whole blood concentrations of ATP and/or its metabolites, and to compare this to direct administration of ATP into the duodenum using a naso-duodenal tube. Although the ATP dosages administered in our study (corrected for body weight 55.6 - 83.3 mg/kg) exceed those of other oral administration studies, we observed no changes in erythrocyte ATP concentrations. Kichenin *et al.* orally administered ATP in dosages of up to 20 mg/kg per day to rabbits and 5 mg/kg per day to rats[9, 10]. In humans, ATP dosages ranging from 36 to 108 mg/kg per day were administered intravenously by Haskell *et al.* and Agteresch *et al.*[12, 13, 17]. Of the ATP metabolites considered, only uric acid concentrations increased significantly after administration of the proximal-release pellets and the duodenoal tube, but not of the distal-release pellets. Assuming that uric acid is primarily present in the extracellular fluid (the volume of which is approximately 22% of body weight), that the 5000 mg ATP is completely broken down to 9.06 mmol uric acid, and that there is no loss of uric acid due to excretion, the

estimated 'bioavailability' of ATP (defined as the observed uric acid increase as a percentage of the theoretical maximum) was 16.6 ± 2.3% for the duodenal tube, 14.9 ± 2.5% for the proximal-release pellets and 3.2 ± 0.6% for the distal-release pellets.

When ATP is released in the small intestine, ecto-nucleotidase triphosphatase diphosphohydrolases present on the luminal side of enterocytes dephosphorylate ATP via ADP to AMP[23], after which ecto-5'-nucleotidase (CD73) degrades AMP to adenosine[24]. In mice, the terminal ileum is the site in the intestine with the lowest ATPase activity[25]. Although information on the human intestine is limited, this may explain the difference in plasma uric acid concentrations after ingesting the proximal or distal-release pellets. Concentrative (CNT) and equilibrative (ENT) nucleoside transporters are able to transport nucleosides into the intestinal enterocytes and to the capillary bed of the intestinal villi. CNTs exhibit a proximal-distal gradient with highest transport activities present in the jejunum[26]. Finally, adenosine is taken up by the erythrocytes through ENTs in the erythrocyte membrane[27]. *In vivo* studies in animals and humans indicated that inside the erythrocytes adenosine can be used for the synthesis of ATP[12, 13]. In our study, neither ATP nor adenosine concentrations were increased, suggesting that instead of being used for ATP synthesis in the erythrocytes, orally administered ATP is degraded to uric acid by xanthine oxidase, an enzyme which is expressed mainly in the liver and in endothelial cells of blood vessels[28].

In our study, the increase in plasma uric acid concentration was similar for the proximal-release pellets and the duodenal tube, indicating complete release of ATP from the pellets. The delay in uric acid increase of about 1 h following proximal-release pellet administration compared to duodenal tube administration is probably a combined effect of gastric residence time and the time needed for dissolution of the coating of the pellets. We used enteric pH-sensitive coated pellets because they were previously successfully used for the targeted delivery of various compounds[29-31]. Pellets were preferred over capsules, because they ensure a short and predictable gastric residence time (mean <1 h in fasted state[32]), are easier to swallow[33, 34] and distribute more homogeneously in the gastrointestinal tract, improving the absorption possibilities for large molecules, like ATP[31]. The pH-sensitive Eudragit® polymer coating provided sufficient gastroresistance, as unwanted *in vitro* release of ATP from the pellets was within the limits set by the USP (i.e. <10% drug release in 2 h in 0.1 N HCl)[35]. *In vivo*, the intestinal pH and transit times are the main factors determining the location where each type of coating releases its contents. The duodenum has a pH of 6.4 with a mean transit time to the jejunum of 30 min, while in the ileum, the pH rises to 7.4 with a transit time to the colon for pellet dosage forms in fasted individuals of approximately 3 ± 1 h (mean ± SD)[32, 36, 37]. The modest rise in uric acid concentration after ingestion of the distal-release pellets may be partly caused by incomplete release in the small intestine, in combination with the limited uptake of ATP once it has entered

the colon[38]. Timely release of the contents of the pellets was confirmed by using lithium as a marker. As expected from earlier studies in which lithium was used as a marker[39], the lithium dosage administered to the subjects was safe; the highest plasma lithium concentration amounted to only 17% of the lower therapeutical range advised for patients with bipolar disease[40]. Remarkably, higher lithium concentrations were reached after administration of the placebo pellets compared to ATP pellets. Possibly, the higher content of carboxymethylcellulose (CMC), which promotes pellet disintegration by expanding upon contact with water, in the placebo pellets (nearly 100%), compared to the ATP pellets (nearly 50%), resulted in a quicker release of lithium and hence the higher plasma concentration. Another possibility is that the negative charges on the CMC molecule, which promote its exposure to water, are shielded by the sodium-ions in the ATP pellets, thus slowing the swelling of CMC in the pellets and thereby the release of their contents.

What may be the consequences of increased plasma uric acid concentrations obtained by orally administering ATP? On the one hand, hyperuricemia is a risk factor for gout and is associated with hypertension[41-45]. For instance, the increase in blood pressure of hyperuricemic rats has been shown to be mediated in part by stimulation of the hormonal renin-angiotensin system by uric acid[41].The highest individual uric acid concentration (405 μmol/L) we observed, is within the range reported for male non-gouty individuals (179-440 μmol/L)[46]. At higher concentrations the risk for uric acid crystal formation and precipitation increases, since uric acid becomes less soluble in the extracellular fluid[47]. No adverse effects were observed during the study,. The short-lasting increase in uric acid concentration found in the current study is not likely to cause any symptoms of gout or hypertension, since these require a prolonged period of severe increase[48]. On the other hand, high uric acid concentrations have also been associated with beneficial health effects. For instance, mutations disabling the uricase gene, which have occurred in the course of hominid evolution, may have provided an evolutionary advantage, because uric acid may function as an antioxidant[49, 50]. Epidemiological studies have shown that healthy subjects with high uric acid concentrations are at a reduced risk for developing Parkinson's disease (PD), a condition suspected to be instigated by oxidative damage[51, 52]. Furthermore, patients with multiple sclerosis (MS) are known to have lower uric acid concentrations than healthy volunteers, and raising the uric acid concentration by pharmacological means has been the subject of recent investigation[53]. Capsules containing inosine were administered to MS patients in amounts between 1 and 3 g/day. This was done until serum uric acid levels between 6-9 mg/dL (=416-535 μmol/L) were reached[54]. Oral administration of ATP might be considered as an alternative to inosine for raising uric acid levels in MS patients. Although increasing the uric acid concentration pharmacologically using ATP pellets might have benefits for certain individuals, these have to be weighed against increased risks of gout and possibly cardiovascular disease[41, 44, 45].

Conclusion

Thus, our results show that the effects of a single oral administration of ATP by means of proximal-release enteric coated pellets mimic the effects of administration by a duodenal tube. An increase of 50% in plasma uric acid concentrations, but no increase in concentration of ATP or any of its other metabolites was observed. These results are in contrast with studies showing increased ATP concentrations in erythrocytes after intravenous administration of ATP, and a single dose of oral ATP can therefore not replace intravenous ATP administration. Further studies will be necessary to determine whether prolonged daily administration of oral ATP is needed to raise erythrocyte ATP concentrations.

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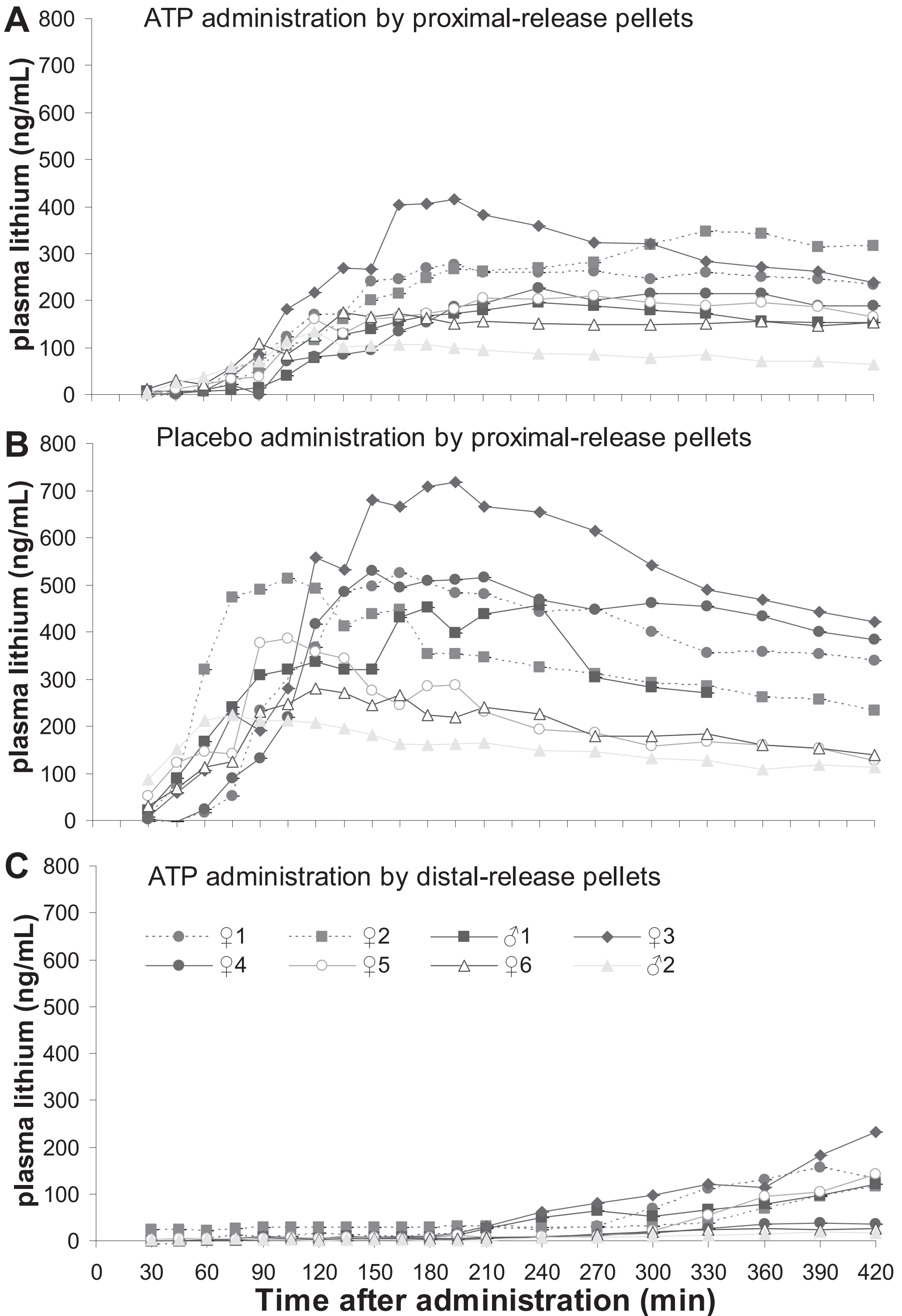
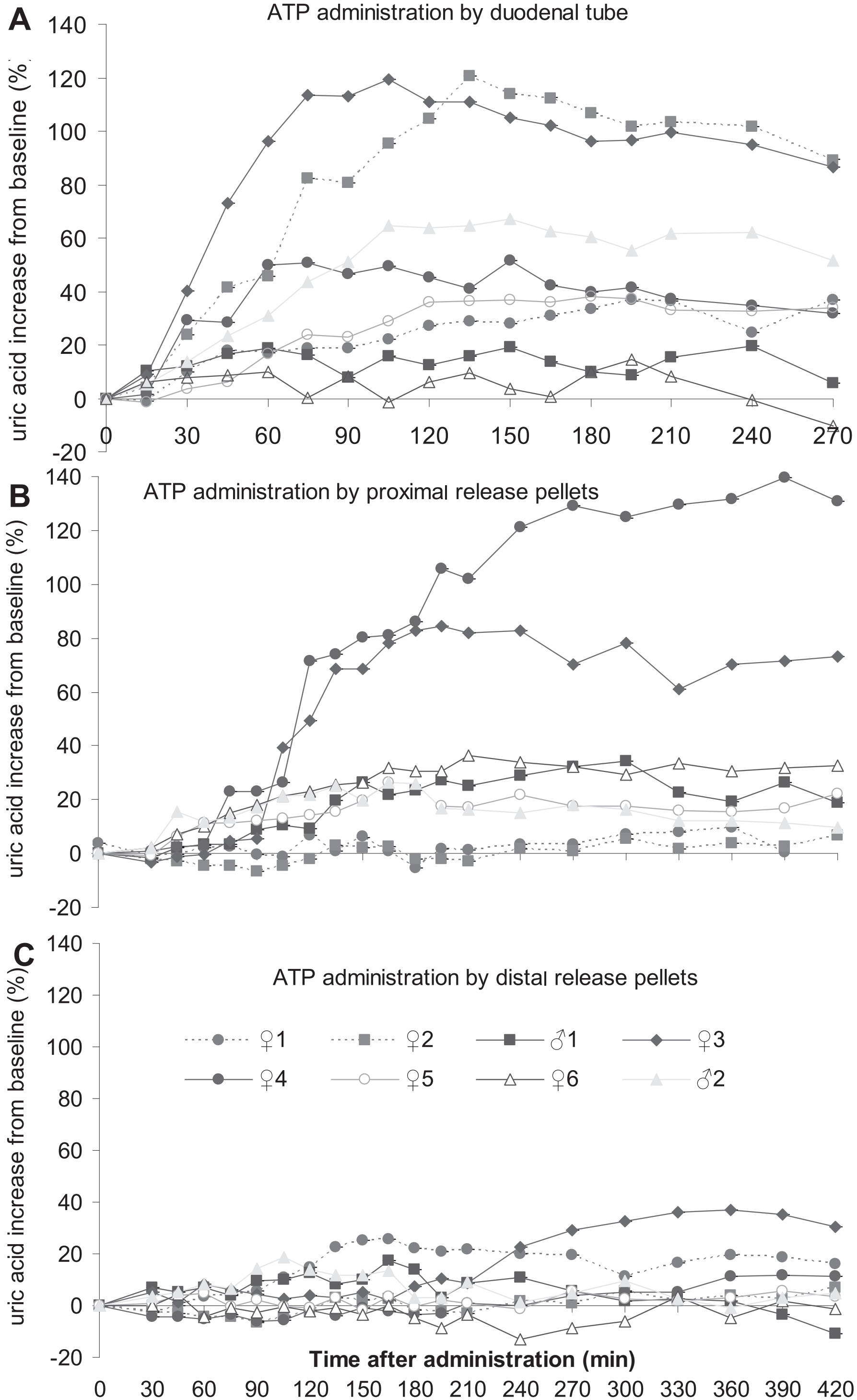


Supplementary Figure 1: Individual concentration-time profiles of plasma lithium in six female and two male volunteers after (A) proximal-release pellets containing ATP, (B) proximal-release pellets containing placebo or (C) distal-release pellets containing ATP.



Supplementary Figure 2: Individual increase in plasma uric acid concentrations following ingestion of 5000 mg ATP at t=0 as a solution through a duodenal tube (A), proximal-release pellets (B), or distal-release pellets (C). Values represent the percentage increase over the mean baseline values that were determined in three samples collected at 30, 20 and 10 min before administration. The legend shows sex of subjects. Note the different scale of x-axis in panel A.

Chapter 4

Oral bioavailability of ATP after prolonged administration

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British Journal of Nutrition (2011), 105: 357–366

Abstract

Purinergic receptors are important for the regulation of inflammation, muscle contraction, neurotransmission, and nociception. Extracellular adenosine 5'-triphosphate (ATP) and its metabolites are the main ligands for these receptors. Occasional reports on beneficial results of ATP administration in human and animal studies have suggested bioavailability of oral ATP supplements. We investigated whether prolonged daily intake of oral ATP is indeed bioavailable. Thirty-two healthy subjects were randomized to receive 0, 250, 1250, or 5000 mg/d ATP for 28 days by means of enteric coated pellets. In addition, on days 0 and 28, all 32 subjects received 5000 mg ATP to determine whether prolonged administration would induce adaptations in the bioavailability of ATP. Four weeks of ATP supplementation did not lead to changes in blood or plasma ATP concentrations. Of all ATP metabolites, only plasma uric acid levels increased significantly after administration of 5000 mg of ATP. Prolonged administration of ATP was safe as evidenced from liver and kidney parameters. We conclude that after oral administration of ATP only resulted in increased uric acid concentrations. Based on these findings, we seriously question the claimed efficacy of oral ATP at dosages even lower than in this study.

Introduction

Next to its intracellular role as an energy carrier, adenosine 5'-triphosphate (ATP) and its metabolites function extracellularly as the main ligands involved in purinergic signalling. Purinergic signalling is important for the regulation of inflammation, muscle contraction, neurotransmission, and nociception (see [1-3] for recent reviews). Modulation of purinergic signalling by administration of purine receptor antagonists (e.g. clopidogrel[4]), or by intravenous administration of ATP or adenosine[5, 6] has been shown to effectively modulate human health.

Despite the small number of studies on the effectiveness of oral ATP administration to modulate purinergic signalling, capsules and tablets containing ATP at dosages between 90 and 250 mg/d are being marketed on the internet as being highly efficacious. Capsules containing 30 mg ATP are currently registered in France (Atépadène®) as an adjunct in the treatment of low back pain of muscular origin. In the late 1980s, two randomized, double-blind, placebo-controlled trials were published in non-indexed medical journals, showing that administration of 90 mg Atépadène® was safe[7, 8]. In a recent double-blind trial, the efficacy of Atépadène® for one month compared to placebo was investigated in patients with low back pain[9], while in an open-label trial, the same drug was tested against the advice to stay active[10]. Both trials indicated that oral ATP had a modest benefit on a secondary endpoint (the use of rescue analgesics) when compared with the non-pharmacological treatment. No differences between ATP and placebo groups were

found when considering pain or functional status of the patients. The investigators also noted that in the non-blinded trial, the positive effect might largely be explained by the patients' knowledge of receiving a drug[9, 10].

In order to investigate the bio-availability of oral ATP, we previously conducted a study in which an acute oral dose of ATP was administered as an enteric coated multi-particulate formulation designed to release its contents in the proximal small intestine (further referred to as "pellets")[11]. This single dose of ATP pellets did not result in any increase in blood concentrations of ATP or of its metabolites except uric acid. Results remained similar when ATP was administered directly into the duodenum via a naso-duodenal tube. We concluded from this study that a single dose of oral ATP at the given dosage is not effectively taken up. So far, the oral bioavailability of ATP after prolonged administration (as recommended for oral ATP supplements on the internet) has so far only been studied in animals. In experimental studies, contrary to expectations, basal plasma ATP levels in animals treated with 5 mg/kg orally administered ATP for 30 days were lower than those in water-treated control animals, suggesting potential up-regulation of ATP-metabolizing enzymes in plasma over the 30-day study. A similar effect was found when oral ATP doses of 1, 5 or 10 mg/kg were given for 20 days. Interestingly, when a single dose of [^{14}C]-labelled ATP was administered directly into the lumen of the jejunum of the rats treated with ATP for 30 days, a rapid 1000-fold increase in portal plasma ATP concentration was found compared to control rats[12]. The authors suggested this to be the result of breakdown of ATP to adenosine by intestinal nucleotidases followed by increased absorption of adenosine by nucleoside transporters present in the intestinal membrane, in combination with an increased efflux of ATP and nucleosides from intestinal cells into the blood stream. Several nucleoside transporters are now known, belonging to two families of concentrative and equilibrative nucleoside transporters (CNT and ENT, respectively)[13]. To test whether such an adaptive metabolic response to chronic ATP administration as observed in animals might also occur in humans, we set out to study this in healthy human volunteers.

The present study had two specific objectives: first, to establish whether whole blood and plasma concentrations of ATP and its degradation products are influenced by oral administration of 0, 250, 1250 or 5000 mg/day ATP for 4 weeks in healthy humans; and second, to investigate whether 4 weeks of oral ATP administration leads to adaptations in uptake, metabolism, and excretion of a single acute 5000 mg dose of ATP. Adverse events were registered and safety was monitored by measuring a set of liver and kidney blood parameters.

Materials and methods

Study design

A total of 33 subjects were assigned in a blinded manner to one of four dosage groups by computer-generated randomization lists with a block size of 8. On days 1-27, subjects were instructed to ingest enteric-coated pellets twice a day, delivering a daily dose of ATP of either a) 5000 mg, b) 1250 mg, c) 250 mg or d) 0 mg (placebo). On days 0 and 28, all subjects received a single dose of 5000 mg ATP as enteric-coated pellets. Subjects ingested the pellets with approximately 200 ml of water that had been acidified to a pH between 1 and 5.

Participants

Healthy male and female volunteers were recruited. Exclusion criteria were: history of lung, heart, intestinal, stomach or liver disease, use of prescription medication (except contraceptives), smoking, drug use, dietary restrictions and pregnancy. Furthermore, only subjects aged 18-60 y could participate. To minimize between-subject variation, subjects were requested to abstain from products containing alcohol or caffeine as well as from purine-rich foods, such as game, offal, sardines, anchovies and alcohol-free beer for two days before test days 0, 7, 14, 21, and 28. Subjects were asked to fast from 10 p.m. the previous day until the end of test days 0 and 28 (4 p.m.), and to refrain from any vigorous physical activity 24 h before each test day. On days 0 and 28, subjects were allowed to drink water starting 30 min after ATP administration. On all intervening days, subjects were instructed to ingest the pellets at two self-chosen moments during the day, each one either at least half an hour before or 2 hours after a meal. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of Maastricht University Medical Centre (MUMC), The Netherlands. Written informed consent was obtained from all subjects.

Materials

ATP disodium salt and adenosine were purchased from Fagron BV, Uitgeest, The Netherlands. Adenosine diphosphate (ADP) disodium salt, adenosine monophosphate (AMP) sodium salt, adenine, inosine, hypoxanthine, uric acid, and perchloric acid (PCA) 70% solution in water were purchased from Sigma Chemical Co., St. Louis, USA. KOH, KH2PO4, K_2CO_3, $K_2HPO_3*3H_2O$ and NaOH were obtained from Merck, Darmstadt, Germany, and 0.9% saline was purchased from Braun, Melsungen, Germany.

Production of pellets

Pellets were obtained from the laboratory of Pharmaceutical Technology, Ghent University, Ghent, Belgium. ATP pellets were produced as described by Huyghebaert et al.[14], with minor modifications to obtain an ATP content of >40%

(wt:wt) after coating. Placebo pellets were produced in the same manner, but without ATP. The pellets were coated with 30% Eudragit® L30D-55 (Röhm Pharma, Darmstadt, Germany) and mixed with anionic copolymers of methacrylic acid and ethylacrylate (1:1). After coating, the pellets were cured overnight at room temperature at 60% humidity, packed in sealed double-plastic bags, and stored at 4°C. Pellets were used within 3 months after production. To ensure blinding of all personnel involved in the study, a separate person not otherwise involved in the study mixed the ATP and placebo pellets at proportions needed to obtain the different doses (0, 250, 1250 and 5000 mg ATP/day), packaged and sealed the pellets in small plastic bags each containing one daily dose, and coded them with subjects' ID-numbers according to the randomization list.

Dissolution testing

The pellets underwent quality control using a dissolution test as described before(11). This test confirmed that the dissolution properties of the pellets were similar to those used in our previous study(11). The release of ATP or degradation products after 120 min in 0.1 N HCl was below 3%. Subsequent transfer to a buffer solution of pH 6.5 caused a release of 50% of the remaining ATP within 10 min, which increased to >90% after 60 min (data not shown).

Sample collection

On days 0 and 28, subjects arrived at the study facility (MUMC+) at around 8 a.m. Venous blood was collected from the antecubital vein using a 20 gauge intravenous catheter (Terumo-Europe NV, Leuven, Belgium), connected to a three-way stopcock (Discofix®, Braun Melsungen AG, Melsungen, Germany). Blood was collected into 4 mL EDTA tubes (Venosafe, Terumo-Europe NV) by inserting a 21 gauge multisample needle (Venoject Quick Fit, Terumo-Europe NV) into the membrane of a closing cone (IN-Stopper, Braun Melsungen AG) that was attached directly to the stopcock. The anticoagulant EDTA inhibits the extracellular hydrolysis of ATP by Ca^{2+}- and Mg^{2+}-activated enzymes, such as plasma membrane-bound CD39(15). Serum tubes were used to collect samples for assessment of safety parameters (see below for details). After blood collection, the tubes were gently inverted three times and put on ice. For whole blood measurements, 500 μL EDTA blood was immediately added to 500 μL ice-cold PCA (8% wt:v), vortex-mixed and frozen in liquid nitrogen. For plasma ATP determination, every second sample of EDTA blood was diluted 1:2 with FireZyme Dilution Buffer (BioMedica Diagnostics Inc., Windsor NS, Canada), and immediately centrifuged at 3,000 x g for 10 min at 4 °C. All blood samples were stored at -80 °C awaiting analysis.

On days 7, 14, and 21, single samples of venous blood were collected into EDTA tubes for whole blood ATP determination. For the experiments on days 0 and 28, baseline blood was sampled 30, 20, and 10 min before administration of 5000 mg ATP to all 32 subjects. Between 30-210 min after ATP administration, blood samples were taken every 15 min, and between 210-420 min samples were taken

every 30 min. Urine was collected 20 minutes before and 120, 220 and 400 minutes after ATP administration stored on ice before being frozen in -80°C at the end of the day. Total urine volume at each time point was determined and each sample stored separately.

ATP measurement in whole blood samples by HPLC

Equipment, sample preparation and measurement conditions for the determination of ATP and its metabolites ADP, AMP, adenosine, adenine, inosine, hypoxanthine, and uric acid have been previously described and validated (16). Briefly, after thawing, the protein fraction was precipitated (12,000 x g, 10 min, 4 °C) and 40 μL 2 M K2CO3 in 6 M KOH was added to 650 μL of the supernatant to neutralize the pH. The resulting insoluble perchlorate was removed by centrifugation (12,000 x g, 10 min, 4 °C), and 40 μL of supernatant was mixed with 160 μL of 0.05 M phosphate buffer pH 6.0 in HPLC vials. The compounds were quantified with HPLC by UV detection at 254 nm.

ATP measurement in plasma samples

The diluted plasma samples were thawed and a CellTiter-Glo® ATP-assay kit (Promega Benelux BV, The Hague, The Netherlands) was used according to the manufacturers' instructions to measure the bioluminescence on a Glomax plate reader.

Uric acid and creatinine measurement in urine samples

Urine samples were thawed and uric acid and creatinine measured using an automated analyzer (Beckman-Coulter, Sharon Hill, PA, USA). Uric acid concentration were corrected for creatinine by dividing the uric acid concentration by the creatinine concentration.

Safety Parameters

In order to assess the safety of a 4-week period of daily administration of pellets containing up to 5000 mg ATP, concentrations of creatinine, alkaline phosphatase, alanine aminotransfrase (ALAT), lactate dehydrogenase (LD) and creatine kinase (CK) were measured in undiluted serum samples using a Beckman Synchron CX5 (Beckman Instruments, Inc., Gladesville, CA, USA) automatic analyser.

Statistical analysis

Results are reported as mean and standard error of the mean (SEM). Fasting whole blood ATP and metabolite concentrations were calculated from one sample taken in the morning on days 7, 14, and 21, and from the average of three samples taken at 30, 20, and 10 min before administration on days 0 and 28. The change in area under the curve (delta AUC) from the fasting concentration on day 0 was calculated using the trapezoidal rule for each subject for the 28-day period. Delta AUC on days 0 and 28 during the 420 min following ATP administration were calculated relative to baseline values on the same day. For uric acid in urine and for

plasma ATP, the AUC was calculated using the trapezoidal rule. Differences in delta AUC or AUC between dosage groups were evaluated with one-way ANOVA, and an LSD post-hoc test when appropriate. Differences in response to 5000 mg ATP between days 0 and 28 were evaluated with a paired t-test. All analyses were performed using SPSS 17.0 (SPSS Inc. Chicago, USA), and a *p*-value <0.05 was considered statistically significant.

Results

Study population

A total of 32 healthy volunteers (13 men, 19 women) completed the 4-week intervention study. Men were on average 28.8 ± 14.1 (mean ± SEM) years old, and weighed 69.4 ± 9.2 kg with a BMI of 21.8 ± 1.5 while women were on average 21.7 ± 4.6 years old, and weighed 66.7 ± 6.5 kg with a BMI of 22.8 ± 1.6. One female participant (assigned to the placebo group) dropped out of the study because of repeated vomiting after ingesting the pellets. All other subjects tolerated the daily administration generally well. Temporary nausea without vomiting occurred in two women between 120 and 135 minutes after ingestion of the first 5000 mg dose of ATP on day 0 of the study. Other minor remarks included a bad taste of the pellets (n=1), and a diminished appetite (one female subject from the highest dosage group).

ATP and metabolites in whole blood

No significant change in fasting whole blood ATP concentration occurred over the entire 4-week intervention period, using doses of 0 - 5000 mg/d of ATP (Table 1). Administration of a single oral dose of 5000 mg ATP on days 0 and 28 of the study also did not lead to increased ATP concentrations in whole blood during the 420 min following administration in any of the groups, and there was no difference in response to ATP between days 0 and 28 (data not shown). The concentrations of adenosine and other metabolites, except uric acid, were not increased after oral administration (data not shown). Uric acid concentrations increased significantly over the 4-week period, and this was only observed in the 5000 mg dosage group versus the 0, 250 and 1250 dosage groups (Table 1). On days 0 and 28, administration of a single dose of 5000 mg ATP increased uric acid concentrations on average up to 40% above fasting concentrations (Figure 1). Uric acid concentrations started to increase at approximately 30 minutes after ATP administration, and levelled off at approximately 150 minutes until at least 300 minutes after ATP administration. The delta AUC for uric acid over the 420 min period following administration of the single dose of 5000 mg ATP did not differ significantly between days 0 and 28, nor between dosage groups (Table 2).

TABLE 1: WHOLE BLOOD CONCENTRATIONS OF ATP AND URIC ACID IN OVERNIGHT-FASTED HEALTHY SUBJECTS AFTER ORAL ADMINISTRATION OF PLACEBO, 250, 1250, OR 5000 MG/D ATP AS ENTERIC COATED PELLETS FOR 4 WEEKS

Dosage	Day 0		Day 7		Day 14		Day 21		Day 28		Delta AUC[a]	
ATP concentrations (µM)												
0 mg	407	±59	382	±64	423	±72	544	±125	395	±66	209	±1240
250 mg	350	±45	370	±44	357	±39	397	±26	364	±32	-91	±760
1250 mg	419	±36	410	±32	420	±39	415	±37	361	±39	-200	± 326
5000 mg	388	±42	386	±36	406	±27	396	±32	351	±31	754	± 723
Uric acid concentrations (µM)												
0 mg	297	±35	266	±20	298	±31	418	±88	286	±30	364	±828
250 mg	290	±26	319	±15	324	±14	420	±68	302	±18	496	±464
1250 mg	337	±29	365	±31	346	±33	362	±12	316	±22	1	±177
5000 mg	269	±16	348	±18	357	±25	436	±55	317	±11	2536	±694[b]

Data are mean ± SEM values of eight subjects per dosage group.

[a] A significant overall difference in AUC between dosage groups is observed for uric acid (p=0.02), but not for ATP (p=0.83, ANOVA).

[b] Dose of 5000 mg is significantly different from 0, 250 and 1250 mg dosage groups (p<0.03, post-hoc LSD-test).

TABLE 2: CHANGE IN AREA UNDER THE CURVE FROM BASELINE (DELTAAUC) FOR WHOLE BLOOD URIC ACID CONCENTRATIONS ON DAYS 0 AND 28, AFTER A SINGLE ORAL DOSE OF 5000 MG ATP AS ENTERIC COATED PELLETS

Dosage	Day 0		Day 28		P-value
	Mean	SEM	Mean	SEM	
0 mg	5484	518	5321	520	*.828*
250 mg	5364	433	5487	320	*.820*
1250 mg	6076	426	5965	423	*.857*
5000 mg	5403	165	4780	244	*.166*
P-value	*.337*		*.613*		

Data are mean ± SEM values of eight subjects per dosage group. P-values are calculated by paired samples *t*-tests across days, and by oneway ANOVA across dosages.

Plasma ATP

Figure 2 shows that plasma ATP concentrations after administration of 5000 mg ATP on days 0 and 28 did not differ significantly between the different dosage groups (p=0.42 for day 0 and p=0.47 for day 28), nor between days 0 and 28 (p>0.05).

Uric acid and creatinine in urine

Analysis of urine samples collected on days 0 and 28 of the study revealed no clear upward or downward trend in uric acid concentrations relative to creatinine (Figure 3). There was no significant difference between days 0 and 28 within each dosage group (p>0.10 for all dosage groups). Within days 0 and 28, there were no differences between the dosages of ATP (p=0.92 on day 0 and p=0.22 on day 28).

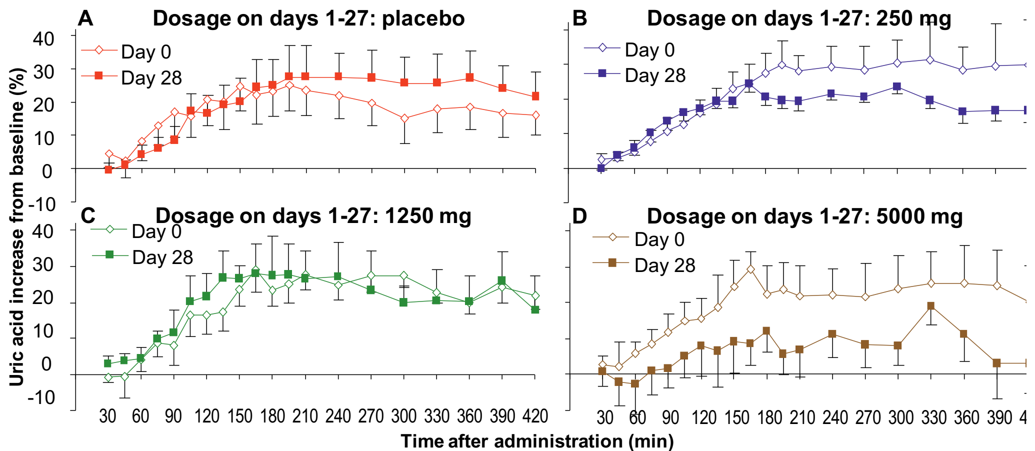


Figure 1: Increase in whole blood uric acid concentration following an acute oral bolus of 5000 mg ATP as enteric coated pellets to healthy subjects. N=8 for each dosage group. On days 1-27, subjects received placebo (panel A), 250 mg/d ATP (B), 1250 mg/d ATP (C), or 5000 mg/d ATP (D).

Safety parameters

As shown in Table 3, none of the safety parameters changed in response to administration of up to 5000 mg ATP for 4 weeks, and all median concentrations remained in the reference range for healthy volunteers. Although we observed some individual values for alkaline phosphatase, lactate dehydrogenase and creatine kinase above the reference range, this occurred to a similar extent at baseline (i.e. prior to ATP/placebo administration) and after 28 days, and also occurred to a similar extent in the placebo and the highest ATP dose group (Table 3).

Discussion

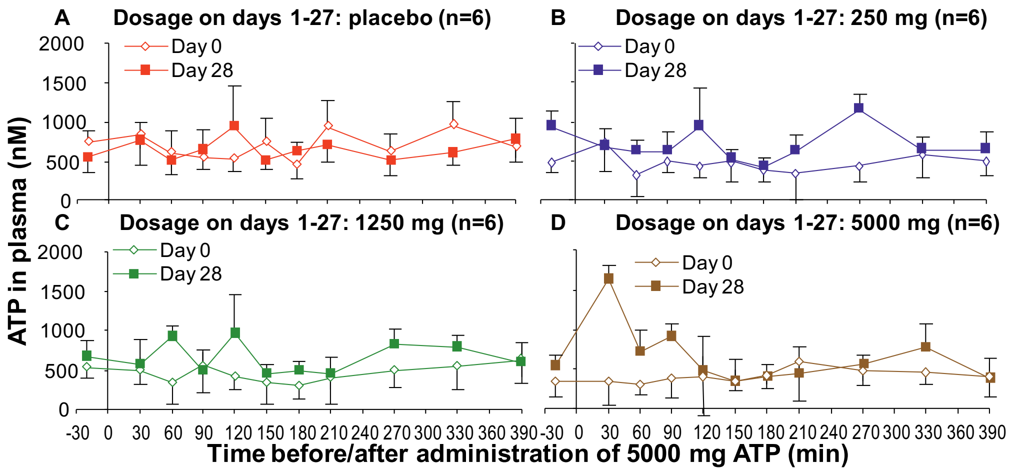
In this randomized double-blind placebo-controlled trial, we investigated the effects of a four-week period of daily oral ATP administration. The first objective of this study was to assess whether prolonged daily oral administration of ATP would lead to increased blood and plasma concentrations of ATP and its degradation products. Even after 4 weeks of oral administration, we detected no rise in concentrations of ATP or any of its metabolites, except for uric acid which showed a significant overall increase in the highest ATP dosage group compared to the other dosage groups over the 4-week period.

Our findings partly agree with previous studies conducted in rats (5 mg/kg/day for 30 days) and rabbits (20 mg/kg/day for 14 days) by Kichenin and colleagues[12, 17] who found no increase in erythrocyte ATP and adenosine concentrations. However, these authors even reported a decrease in basal plasma ATP concentrations of rats receiving 5 mg/kg/day for 30 days [12]. High-dose oral ATP administration trials in humans have not been reported before. In one previous trial, healthy males ingested much lower doses of ATP (0, 150 or 225 mg/d) for 14

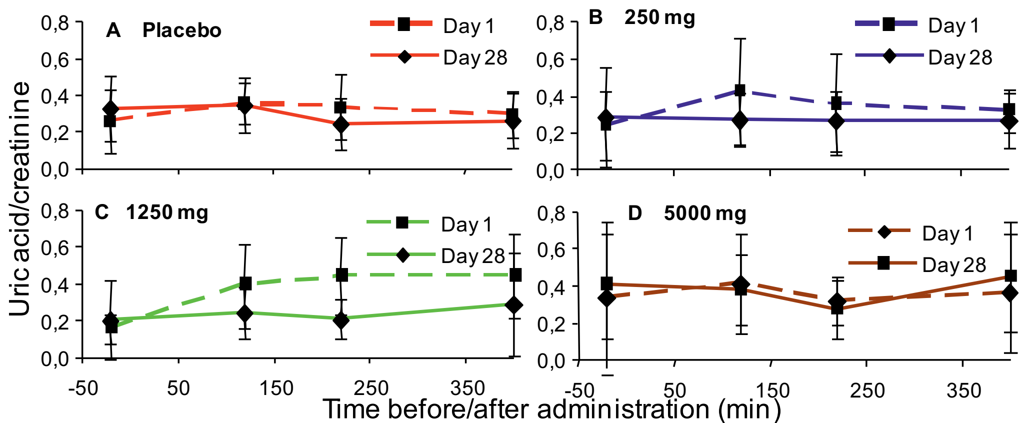
TABLE 3: SERUM CONCENTRATIONS OF FIVE SAFETY PARAMETERS IN SAMPLES COLLECTED 20 MINUTES BEFORE (T=–20) OR 105 MINUTES (T=105) AFTER ORAL ADMINISTRATION OF 5000 MG ATP AS ENTERIC COATED PELLETS TO HEALTHY SUBJECTS.
(MEDIAN VALUES AND RANGES)*

	Dosage on days 1-27															
	Placebo				250 mg				1250 mg				5000 mg			
	t = – 20 min		t = 105 min		t = – 20 min		t = 105 min		t = – 20 min		t = 105 min		t = – 20 min		t = 105 min	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Day 0																
Creat.†	85	62-97	75	59-90	83	64-103	78	63-97	85	67-99	81	71-97	85	65-91	77	63-94
Alk. P†	58	37-140	57	41-145‡§	74	57-112	70	57-119	61	37-93	62	35-84	44	35-68	45	29-67
ALAT†	12	9-19	16	12-20	13	6-39	16	10-40	17	5-19	12	7-17	12	3-16	12	4-16
LD†	289	271-468	340	272-570‡	344	241-437	373	320-754‡|	380	222-767‡§	362	298-447	349	255-511‡§	339	251-407
CK†	65	35-162	62	31-155	83	36-287	139	42-277	67	39-152	74	38-150	74	42-90	86	39-123
Day 28																
Creat.†	76	59-103	75	58-92	78	66-109	74	66-106	86	71-98	84	68-94	79	62-99	76	64-93
ALAT†	49	40-140	48	36-141	80	58-106	77	60-108	54	34-70	54	36-66	67	31-79	50	35-78
Alk. P†	14	11-25	11	5-27	16	6-41	16	5-37	18	8-21	13	11-17	13	10-21	14	5-18
LD†	319	244-555‡|	320	236-510‡§	417	291-609‡¶	366	283-527‡§	395	291-451	354	213-440	394	222-647‡§	397	306-564‡§
CK†	54	33 -241	54	30-220‡|	66	37-329‡¶	61	39-180	99	61-121	82	34-138	97	46-167	86	44-124

Creat, creatinine; ALAT, alanine amino transferase; Alk. P, alkaline phosphatase; LD, lactate dehydrogenase; CK, creatine kinase
* Eight subjects/dosage group
† The reference values for healthy volunteers are as follows: creatinine 60–115mmol/l (male), 50–100mmol/l (female); alkaline phosphatase ,140 IU/l; ALAT IU/l (male), ,35 IU/l (female); LD ,480 IU/l; creatine kinase ,225 IU/l (male), ,160 IU/l (female)
‡ An individual maximum value exceeded the reference value.
§ The number of individuals with higher than maximum values (n=1).
| The number of individuals with higher than maximum values (n=2).
¶ The number of individuals with higher than maximum values (n=4).



Figuur 2: Plasma ATP concentrations following an acute oral bolus of 5000 mg ATP as enteric coated pellets to healthy subjects. ATP was administered at t=0. N=6 or 4 per dosage group. On days 1-27 subjects received placebo (panel A), 250 mg/d ATP (B), 1250 mg/d ATP (C), or 5000 mg/d ATP (D).



Figuur 3: Concentrations of uric acid relative to creatinine in urine following an acute oral bolus of 5000 mg ATP as enteric coated pellets to healthy subjects. N=8 for each dosage group. On days 1-27 subjects received placebo (panel A), 250 mg/d ATP (B), 1250 mg/d ATP (C), or 5000 mg/d ATP (D).Error bars represent SD.

days. No significant increase in ATP levels was observed, and no other metabolites were measured in that human trial [18].

It is known that ATP is quickly metabolised in the small intestine. First, ecto-nucleotidase triphosphatase diphosphohydrolases (NTPDases) present on the luminal side of enterocytes dephosphorylate ATP via ADP to AMP [19]. Second, AMP is also not likely to stay intact in the small intestine, since ecto-5´- nucleotidase (CD73) and alkaline phosphatases degrade it to adenosine[20]. Adenosine can be transported to the intestinal villi and into erythrocytes by nucleoside transporters[21]. We suggest that the enzymes adenosine deaminase (present on endothelial cells) and xanthine oxidase (present in the liver) efficiently degraded adenosine to

uric acid[22]. This could explain our observation that exclusively uric acid was found to be increased during ATP administration. This increase in uric acid, but not in ATP or other metabolites, upon administration of a single dose of 5000 mg ATP is in accordance with our earlier findings [11]. Uric acid has an elimination half-life of approximately 20 hours [23]. Indeed, the uric acid concentration we observed in our study did not decline much during 6 hours after administration of 5000 mg ATP. Since pellets were ingested twice a day from days 1 to 27, the periods between ATP ingestion were close to 12 hours; therefore, partial transfer of uric acid from the last dosage from the previous day may have contributed to the observed rise in basal uric acid concentrations on days 7, 14, 21 and 28 (Table 1). The implications of this increase in uric acid concentration are discussed below.

Our second objective was to investigate whether the uptake, metabolism, and excretion of an acute oral ATP bolus would be influenced by prolonged administration (4 weeks) of 250, 1250, or 5000 mg per day of oral ATP. For this purpose, we performed an experiment with a single acute dose of 5000 mg ATP on day 0, and repeated this experiment in all subjects on day 28. Results showed that, again, only the concentrations of uric acid, and not of ATP or any of the other metabolites, increased immediately after the single dose of 5000 mg ATP. Furthermore, there were no significant differences in concentration changes between days 0 and 28 for any of the measured compounds. Although not significant, the data presented in Table 2 and Figure 1D suggest that in the highest dosage group (5000 mg ATP), the rise in uric acid concentrations in response to administration of 5000 mg ATP on day 28 was smaller than on day 0. Since we found no increase in uric acid excretion in the urine samples collected on day 28 (Figure 3), a more efficient excretion of uric acid is not likely to be the cause.

Previous studies in animals showed that, while systemic concentrations did not change, concentrations of ATP and its metabolites in the portal vein increased after prolonged ATP administration. Clearly, in our healthy volunteers we could not sample portal blood to clarify the intestinal fate of ATP [12].

With this study, we intended to explore whether oral administration of ATP by enteric-coated pellets could be used as an alternative to other means of ATP administration. Intravenous administration of ATP has been described to elicit marked health benefits and promote survival in cancer patients[5, 6, 24-31]. Although it is feasible to intravenously administer ATP at home, some drawbacks include the increased risk of side effects, difficulty of achieving venous access and the need for medical supervision[5, 6, 24-31]. Previous studies with oral administration of ATP have either employed ATP in solution or as relatively large enteric-coated capsules. The latter has the drawback of being difficult to target a specific area of the small intestine due to gastric retention and unreliable enteric coating [18]. In the present study, we prevented these problems by using small enteric-coated pellets with a well-defined release profile, which in addition was tested in pilot experiments. Although the pellets were well-tolerated and did release their contents, we could not

demonstrate any increase in systemic ATP levels in contrast with intravenous ATP administration.

Our results have important implications. First, the reported efficacy of oral ATP supplements that are available through web stores or as registered drugs should be reconsidered. As we find no change in whole blood ATP concentrations at dosages several times exceeding the dosages administered via currently used oral ATP supplements, our results justify strong doubts regarding claims on these supplements as being effective in providing ATP to the body. Second, the observed increases in uric acid concentrations in the present study may well explain some of the physiological effects reported after oral ATP administration in previous studies. Uric acid has a controversial reputation: it is at the same time thought to be 1. an important physiological antioxidant that reduces oxidative stress; 2. a normally innocent metabolic waste product that only causes gout or kidney stones if present in excessively high concentrations; and 3. a causal factor for hypertension, obesity, and vascular and renal diseases [32-35]. With regard to the first hypothesis, there are indications that uric acid may have anti-inflammatory activities in conditions such as multiple sclerosis, Parkinson's disease, and acute stroke [36-38]. These may be partly due to the strong antioxidant capacity of uric acid. The use of oral ATP-containing supplements to increase uric acid concentrations might be of therapeutic value for these diseases. Second, the pro-inflammatory properties of uric acid crystals have long been known to be involved in gout. The uric acid deposits that occur predominately in joints after prolonged periods of excessively high concentrations of uric acid, can eventually trigger an inflammatory response [39]. Uric acid is also known to act as a 'danger signal,' when it is released from necrotic cells [40]. Hyperuricemia is often defined as UA > 7.1 mg/dl (420 μmol/L) in men and > 6.1 mg/dl (360 μmol/L) in women[41, 42]. In our study, the blood concentrations of uric acid stayed below these values. Our data on creatinine, alkaline phosphatase, ALAT, LD and CK also demonstrate that daily administration of 5000 mg ATP is safe. As for the third hypothesis, given the complex relationship of uric acid with other established cardiovascular risk factors, such as obesity, metabolic syndrome, diabetes, and especially hypertension, and the lack of prospective studies, it remains unclear whether uric acid is indeed a causal factor for these conditions[43, 44].

In conclusion, our study shows that a 4-week period of daily administration of ATP as enteric coated pellets does not lead to increased plasma or blood ATP concentrations, nor is there any evidence of a metabolic adaptation in the uptake or metabolism of ATP. Our results cast serious doubt on the health claims of oral ATP supplements.

Acknowledgements. All the authors have contributed significantly to the work. Specification of the authors' contribution to the supplied manuscript: Erik J. Coolen: writing, lab analyses, medical approval and the volunteer study; Ilja C Arts: drafting the manuscript; Otto Bekers: measurements; Chris Vervaet: advice on and production of the pellets used; Martien A Cohen Stuart: drafting the manu-

script, advice; Aalt Bast: manuscript preparation, advice; Pieter C Dagnelie: manuscript preparation, drafting, advice.

All authors state that there were no conflicts of interest.

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors

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Chapter 5

Time-dependent effects of ATP and its degradation products on inflammatory markers in human blood *ex vivo*

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Immunobiology (2008) 213: 389-397

Abstract

We recently reported that adenosine 5'-triphosphate (ATP) modulates cytokine release in lipopolysaccharide (LPS)-phytohemagglutinin (PHA)-stimulated blood. ATP inhibited tumor necrosis factor-alpha (TNF-α) release via activation of the P2Y11 receptor and increased interleukin (IL)-10 release via stimulation of the P2Y12 receptor. Because ATP is known to be broken down by various ecto-enzymes, we determined the degradation profile of ATP in time in LPS-PHA-stimulated blood. ATP slowly metabolized with 14% remaining after 6 h. Simultaneously, adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and hypoxanthine were formed. Subsequently, we investigated the time-dependent effects of ATP and its metabolites on inflammatory markers. Results showed that ATP decreased the rise in concentrations of TNF-α, interferon-gamma (IFN-γ) and IL-1β, but increased concentrations of IL-8 and IL-10. Metabolites of ATP showed either no, similar or opposite effects on cytokine release, compared to ATP. In conclusion, ATP has rapid immunomodulatory effects on a variety of cytokines in stimulated whole blood that persist until 24 h.

Introduction

Purinergic receptors are known to mediate the variety of effects induced by extracellular adenosine 5'-triphosphate (ATP)[1, 6, 26]. Extracellular ATP modulates cytokine release induced by several inflammatory stimulators (lipopolysaccharide (LPS), cytokine mix, etc.) in different cell lines[5]. However, ATP is metabolized by various ecto-enzymes[37] to adenosine 5'- diphosphate (ADP) and adenosine 5'-monophosphate (AMP). AMP can be further degraded to adenosine, inosine and hypoxanthine and eventually to uric acid.

We showed earlier that ATP modulates cytokine release in LPS-phytohemagglutinin (PHA)-stimulated blood, a model closely resembling the in vivo situation in contrast with most studies done in cell lines, by inhibiting tumor necrosis factor-alpha (TNF-α) and increasing interleukin (IL)-10 release[32]. In a subsequent study[33], we showed that the inhibitory effect of ATP on TNF-α release observed 24 h after stimulation was mediated by activation of the P2Y11 receptor. Since this receptor is preferentially activated by ATP[9, 10, 34, 35], this would indicate a possible direct effect of ATP without the involvement of its metabolites. In contrast, the involvement of the P2Y12 receptor, a receptor activated by ADP[11, 35], in the stimulatory effect of ATP on IL-10 release observed 24 h after stimulation, suggested that this effect was due to its metabolite ADP. At present, it is unclear whether ATP modulates cytokine release at earlier time-points than 24 h after stimulation and, moreover, if ATP is able to modulate the release of other cytokines like interferon-gamma (IFN-γ), IL-1β and IL-8 in stimulated human blood.

In the present study, we intended to further characterize the ATP effects in this LPS-PHA-stimulated blood model and the behavior of ATP in this model. The primary aim of the present study was therefore, first to determine the degradation profile of ATP and, second, to investigate the time-dependent effect of ATP and its metabolites on the release of the cytokines TNF-α, IFN-γ, IL-1β, IL-8 and IL-10 in LPS-PHAstimulated blood. Since the transcription factor nuclear factor kappaB (NFκB) is activated during an inflammatory response[2, 3, 25], we further investigated whether the anti-inflammatory effects of ATP were due to interference with the NFκB pathway.

Material and methods

Chemicals

Purified PHA (HA16) was purchased from Murex, Dartford, UK. LPS (*E. coli* 0.26:B6), ADP, AMP, adenine, adenosine, inosine, hypoxanthine, uric acid and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, USA. ATP was purchased from Calbiochem, USA. Human ELISA kits (TNF-α, IFN-γ, IL-1β, IL-8 and IL-10) were obtained from CLB/ Sanquin, Amsterdam, The Netherlands. RPMI 1640 medium containing L-glutamine was obtained form Gibco, Paisley, UK. Bio-Rad protein assay Dye reagent concentrate was obtained from Bio-Rad Laboratories GmbH, Munich, Germany. All other chemicals were of analytical purity.

Study design

Blood was collected from twelve healthy volunteers (age range 25–36 years; 6 women/6 men) in heparincontaining vacutainer tubes (Vacutainer, Becton- Dickinson, 170 IU). Fresh blood was aliquoted into 6- or 24-well plates and incubated with medium (control) or ATP, ADP, AMP, adenosine, inosine, hypoxanthine or uric acid at $t = -30$ min followed by LPS-PHA incubation at $t = 0$ for 24 h. The ATP concentration in the 30 min pre-incubation period (from $t = -30$ to –0min) was 1000 mM, and the final ATP concentration after LPS-PHA addition ($t = +0$min) over the following 24 h was 300 mM. Samples for analysis of cytokines, NFκB, ATP and its metabolites were taken at time points $t = 0$, 15, 30 min and 1, 2, 4, 6 and 24 h after LPS-PHA stimulation. All the incubations were done at 5% CO_2 and 37 °C as previously described[32]. PHA and bacterial LPS were added to whole blood at a concentration of 1 mg/ml and 10 mg/ml, respectively. After each incubation period, cell-free supernatant fluids were collected by centrifugation (3500 rpm, 10 min at 4 °C) and stored at –20 °C for cytokine analysis and at –80 °C for measurement of the concentrations of ATP and its metabolites. To isolate white blood cells, the pellet remaining after centrifugation of the blood was washed 3 times with erythrocyte lysis buffer (containing NH_4Cl, $KHCO_3$ and EDTA) and placed on ice to lyse the erythrocytes. The white blood cell pellets were surface washed with ice-cold

PBS, centrifuged and the supernatant was discarded. Subsequently, the white blood cell pellets were lysed using an ice-cold lysis buffer as described by Hofmann et al. (1999)[20] and centrifuged at 15,000 rpm for 1 min at 4 °C. The nuclear pellets were resuspended in ice-cold extraction buffer as described by Hofmann et al. (1999)[20]. After 20 min at 4 °C, the nuclear lysates were centrifuged at 15,000 rpm for 2 min at 4 °C and the supernatants, containing the nuclear proteins, were immediately collected and stored at –80 °C until analysis of NFκB and protein concentration.

Cytokine measurement via enzyme-linked immune sorbent assay (ELISA)

All cytokines were quantified using PeliKine Compact human ELISA kits (CLB/Sanquin, The Netherlands) based on appropriate and validated sets of monoclonal antibodies. Assays were performed as described by the manufacturer's instructions.

NFκB and protein measurement

NFκB concentrations were determined in nuclear extracts of white blood cells, according to the manufacturer's instructions (TransAM NFκB p50 transcription Factor Assay Kit; Active Motif Europe, Rixensart, Belgium), using a protein content of 200–300 mg/ml in the nuclear extracts; protein concentrations were determined using the method of Bradford (Biorad), using BSA as a standard. Specific NFκB concentrations in the nuclear extracts were determined using a wild-type oligonucleotide incubation versus a mutated oligonucleotide incubation.

ATP measurement in plasma by high-performance liquid chromatography (HPLC)

The analyses of the concentrations of ATP, ADP, AMP, adenosine, inosine, hypoxanthine and uric acid were done according to the method described by Schweinsberg and Loo (1980)[30] with minor modifications. Plasma samples were deproteinized with a final concentration of 4% (v/v) perchloric acid. The supernatant was neutralized (pH 6–7) with 2M K_2CO_3 in 6M KOH and centrifuged (14,000 rpm, 10 min, 4 °C). In a single run, ATP and its metabolites were quantified using an HPLC system (Agilent, Palo Alto, CA, USA) equipped with a UV/VIS detector (254 nm). Separation was achieved with a 3 mm ODS Hypersil C18 RP column (150 x 4.6mm i.d.; Thermo Electron Corp., USA) protected by a 5 mm Hypersil C18 guard column (10x4mm i.d.; Alltech BV, Breda, The Netherlands). Fifty millimolar phosphate buffer (pH 6.0) (mobile phase A), 100% methanol (mobile phase B) and a flow of 1 ml/min were employed. From 0 to 2 min, a linear gradient was started with 100% mobile phase A, from 2 to 10 min the amount of mobile phase B was linearly increased to 12.5% and remained for 2 min at 12.5%. Finally, the amount of methanol was reduced to 0% and the gradient returned to 100% mobile phase A at 17 min. Concentrations of ATP and its metabolites were calculated by comparing peak areas with appropriate standards.

Statistics

Changes in cytokine release and NFκB activation compared to time point 0 h were appraised by Wilcoxon's signed rank test, and effects of ATP and its metabolites on cytokine release compared to the control condition (medium) were determined using Mann–Whitney U-test. Two-tailed p-values of 0.05 or less were considered statistically significant. Results are reported as means±SEM.

Results

ATP degradation profile in LPS-PHA-stimulated human blood

Steady-state concentrations in plasma before the addition of ATP were <1 μM for ATP, ADP and AMP and 356±81 μM for uric acid. Fig. 1 shows the time-dependent degradation of ATP and the simultaneous formation of its metabolites ADP, AMP and hypoxanthine in human blood at different time points after LPS-PHA stimulation. Other known metabolites of ATP, such as xanthine, adenosine, adenine or inosine were not detected. The molar balance (which is determined as the sum of the molar concentrations of ATP and its metabolites) in the samples remained stable during the experiment. In Table 1, changes in plasma levels of ATP and its metabolites over time are shown, expressed as percent of molar balance. ATP levels decreased from 91% at $t = -30$ min to 78% at $t = -0$ min. The fact that at $t = -30$ min, ATP is only present as 91% of the molar balance, and not as 100% as would be expected, is probably due to the breakdown of ATP during collection and centrifugation of blood samples. After addition of LPS and PHA (i.e. t=+0min), the percentage ATP of the molar balance further declined to 35% after t=+2h; after t = +6h, ATP constituted only 5% of the molar balance. As ATP concentrations declined, both ADP and AMP were formed. ADP increased from 17% at t= +0min to 32% after t =+2h, followed by a gradual decrease to 0.2%. AMP levels increased from 3% of the molar balance at t = +0min to 70% after t = 6h, followed by a decrease after t =+24 h. Hypoxanthine levels showed a gradual rise from t=+2–6 h, with an increase after t =+24 h. Uric acid levels remained constant (data not shown). The ATP breakdown profile in blood not treated with LPS-PHA was similar (data not shown). Furthermore, control experiments showed that no ATP was formed in blood treated with LPS-PHA only (data not shown).

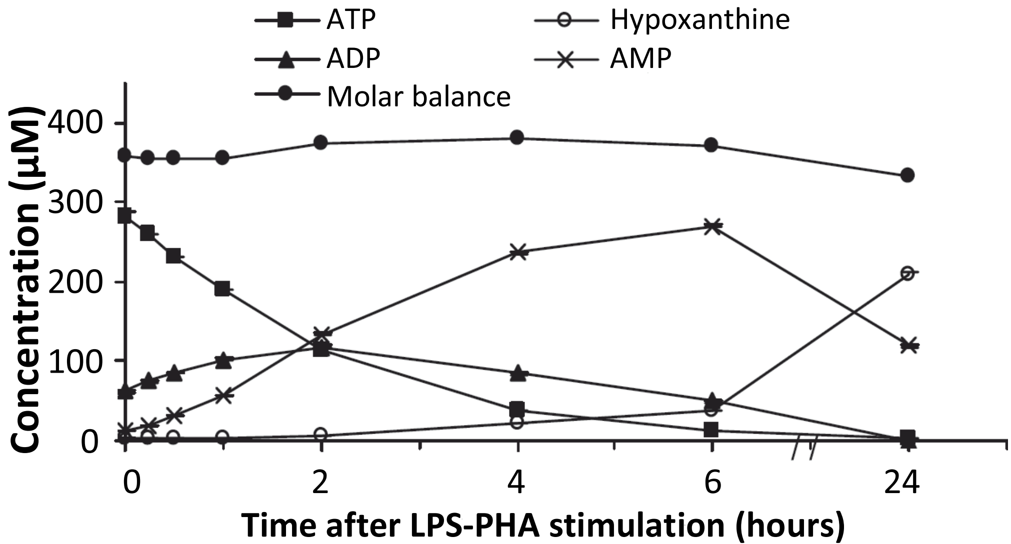

Figure 1. Degradation profile of ATP at different time-points after LPS-PHA stimulation in diluted blood from healthy subjects. The concentration of each of the detected components is plotted on the Y-axis. Curves represent means, and error bars SEM (n = 6).

TABLE 1. PLASMA LEVELS OF ATP AND ITS METABOLITES OVER TIME. EXPRESSED AS PERCENTAGE OF THE MOLAR BALANCE[A]

Time	ATP (%)	ADP (%)	Hypoxanthine (%)	AMP (%)	Molar balance (%)
–30 min	91.08	6.34	2.34	0.22	100.00
–20 min	86.98	9.59	2.45	0.95	100.00
–10 min	82.11	13.71	2.46	1.70	100.00
–0 min	77.44	16.98	2.59	2.97	100.00
+ 0min	78.27	17.51	1.50	2.71	100.00
+15 min	73.59	20.56	0.55	5.31	100.00
+30 min	68.68	23.15	0.66	7.50	100.00
+1 h	56.99	27.98	1.03	14.00	100.00
+2 h	35.26	31.78	1.86	31.10	100.00
+4h	13.49	25.28	5.18	56.04	100.00
+6h	4.90	15.79	9.39	69.90	100.00
+24 h	0.38	0.17	67.07	32.39	100.00

[a]Time points –30 min to –0 min refer to the pre-incubation period. Time-points +0min to +24h refer to the subsequent LPS-PHA incubation period. Thus, t =–0 min refers to the time-point just preceding addition of LPS and PHA, and t = +0min to the time-point after LPS and PHA addition.

Effects of ATP on inflammatory markers at different time points after LPS-PHA stimulation

Figs. 2A–E show the effects of ATP on different cytokines in human blood at 2, 4, 6 and 24 h after LPSPHA stimulation. TNF-α concentrations increased between 2 and 6 h after LPS-PHA stimulation, but decreased thereafter (Fig. 2A). ATP pre-treatment of blood attenuated the rise in TNF-α concentrations at all time points (mean±SEM: 21±3% at 2 h, 41±3% at 4h, 42±2% at 6 h and 58±5% at 24 h).

IFN-γ concentrations started to rise at 4 h after stimulation and continuously increased until 24 h (Fig. 2B). ATP reduced the rise in IFN-γ concentrations at 4 h (56±5%), 6 h (54±5%) and 24 h (72±3%). Concentrations of IL-1β increased from 4 h until 24 h after stimulation (Fig. 2C). Again, pre-treatment with ATP caused a suppression of the increased IL-1β concentrations at 6 h (26±4%) and 24 h (18±4%). As shown in Fig. 2D, LPS-PHA-stimulation induced a continuous increase of IL-8 concentrations from 2 to 24 h after stimulation. Pre-treatment of blood with ATP increased the induced IL-8 concentrations at all time points (23±2% at 2 h, 29±3% at 4 h, 20±5% at 6 h and 11±2% at 24 h). The concentrations of IL-10 were increased from 4 to 24 h after, with a sharp rise between 6 and 24h (Fig. 2E). ATP further increased the induced IL-10 concentrations at 4 h (29±3%), 6 h (22±2%) and 24 h (55±4%). As shown in Fig. 2F, the activation of NFκB increased from 2 to 24 h after LPS-PHA challenge in human blood. Pre-treatment with ATP attenuated this NFκB activation at 2 h only (18±3%). Effects of ATP metabolites on inflammatory markers at different time points after LPS-PHA stimulation

As shown in Table 2, ADP and AMP significantly inhibited the LPS-PHA-induced rise in TNF-α concentrations at 4, 6 and 24 h. Inosine caused an increase in the LPS-PHA-induced TNF-α concentration, which was only significant at 24 h. Adenosine, hypoxanthine and uric acid showed no effect on the LPS-PHA-induced TNF-α levels. LPS-PHA-induced rise in IFN-γ concentrations was significantly decreased by ADP, AMP and adenosine. ADP and AMP showed this effect at 4, 6 and 24 h and adenosine only at 6 and 24 h. Inosine, hypoxanthine and uric acid showed no effect on LPS-PHA- induced IFN-γ. ADP increased the LPS-PHA induced IL-1β release at 6 and 24 h and AMP and hypoxanthine at 24 h. Adenosine, inosine and uric acid showed no significant effect on the induced IL-1β concentrations. The LPS-PHA-induced rise in IL-8 levels were significantly increased by ADP and AMP at 2, 4, 6 and 24 h. Adenosine, inosine, hypoxanthine and uric acid showed no effect on IL-8. The LPS-PHAinduced rise in IL-10 concentrations were only significantly increased by ADP at 24 h. Hypoxanthine, adenosine and inosine significantly decreased the stimulation- induced rise in IL-10 concentrations at 24 h. AMP and uric acid showed no effect on IL-10 concentrations.

Discussion

We recently showed that the natural compound ATP is able to modulate cytokine release in LPS-PHAstimulated blood by simultaneously inhibiting TNF-α release via activation of the P2Y11 receptor and increasing IL-10 release by activating the P2Y12 receptor(32, 33). These data were observed 24 h after LPS-PHA stimulation of blood. So far, it was unclear whether ATP also exerts these immunomodulatory effects at earlier time points, and whether ATP is able to modulate the release of other cytokines, besides TNF-α and IL-10. The combined use of the

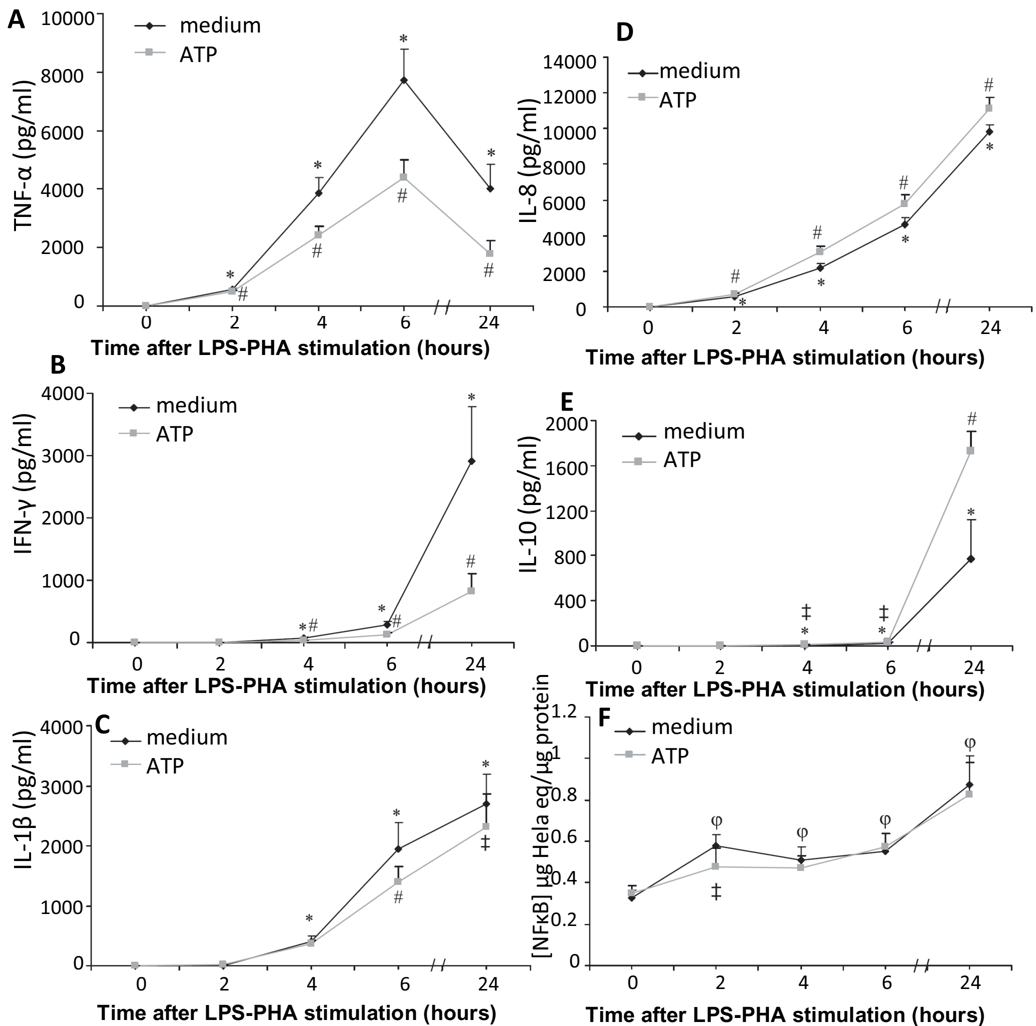

Fig. 2. Modulating effects of ATP (300 µM) on TNF-α (A), IFN-γ (B), IL-1β (C), IL-8 (D), IL-10 (E) concentrations and NFκB (F) activation induced at different time points (0, 2, 4, 6 and 24 h) after LPS-PHA stimulation in blood from healthy subjects. Curves represent means, and error bars SEM (n = 12). * p<0.01 and ϕp<0.05 compared to 0 h after LPS-PHA stimulation. #p<0.01 and ‡p<0.05 compared to stimulation in the absence of ATP.

stimulators LPS and PHA was selected for the simultaneous activation of the innate and the adaptive immune response. In this way, all types of immune cells are activated, leading to the production of a range of different cytokines.

ATP in the extracellular compartment is known to be metabolized by various ecto-enzymes and xanthine oxidase [37]. Several groups have shown that ATP, when added to whole blood, was completely degraded within 30 min[8, 19]. Our results indicate that the complete degradation of ATP, added to LPS-PHA- stimulated blood, takes more than 6 h. This discrepancy can be explained by the fact that we diluted the blood four times with medium, thus lowering ectoenzyme concentrations. There was no difference in the degradation profile of ATP when saline instead of medium

Table 2. Effect of ATP metabolites (300 µM) on cytokine release in LPS-PHA-stimulated blood[a]

TNF-α (pg/ml)	0 h	2 h	4 h	6 h	24 h
Medium	1.47 (0.1)	614 (65)	5001 (703)	9744 (1431)	5181 (1490)
ADP	1.40 (0.06)	622 (86)	3169 (535)*	5696 (810)*	2879 (958)*
AMP	2.00 (0.5)	531 (48)	2873 (502)*	5288 (719)*	2786 (1028)*
Adenosine	1.43 (0.9)	546 (39)	5603 (868)	10633 (1892)	5726 (1454)
Inosine	1.48 (0.1)	664 (39)	6317 (1012)	10789 (2460)	6115 (1667)*
Hypoxanthine	1.51 (0.09)	690 (61)	5914 (969)	8983 (1551)	5210 (1464)
Uric acid	1.40 (0.14)	599 (39)	4677 (729)	8417 (1330)	4952 (965)
IFN-γ (pg/ml)	0 h	2 h	4 h	6 h	24 h
Medium	2.07 (0.25)	2.02 (0.13)	97 (35)	307 (83)	3687 (1530)
ADP	1.74 (0.09)	2.10 (0.15)	49 (19)*	143 (42)*	1190 (528)*
AMP	1.82 (0.13)	1.94 (0.1)	54 (22)*	147 (44)*	1515 (769)*
Adenosine	1.8 (0.14)	2.12 (0.15)	92 (37)	232 (69)*	2020 (805)*
Inosine	1.74 (0.1)	1.99 (0.13)	99 (38)	303 (85)	3167 (85)
Hypoxanthine	1.80 (0.12)	2.05 (0.11)	78 (24)	227 (56)	3107 (1267)
Uric acid	1.73 (0.12)	1.99 (0.14)	87 (32)	261 (73)	2942 (1298)
IL-1β (pg/ml)	0 h	2 h	4 h	6 h	24 h
Medium	2.85 (0.89)	1.33 (0.17)	325 (50)	1271 (360)	1774 (279)
ADP	2.80 (0.85)	1.68 (0.22)	308 (48)	1452 (362)*	2897 (594)*
AMP	2.76 (0.75)	1.34 (0.09)	250 (31)	1090 (263)	2327 (474)*
Adenosine	2.88 (0.89)	1.45 (0.15)	300 (47)	1387 (875)	2980 (1453)
Inosine	2.97 (0.85)	1.29 (0.1)	330 (75)	1213 (343)	2193 (359)
Hypoxanthine	8.10 (5.02)	1.29 (0.11)	344 (49)	1172 (326)	2197 (327)*
Uric acid	2.33 (0.65)	1.24 (0.1)	286 (36)	1089 (236)	1645 (180)
IL-8 (pg/ml)	0 h	2 h	4 h	6 h	24 h
Medium	4.19 (1.39)	480 (87)	2255 (309)	4331 (297)	10772 (262)
ADP	4.13 (0.83)	824 (105)*	3429 (383)*	4963 (165)*	12574 (213)*
AMP	3.96 (0.98)	577 (87)*	3156 (432)*	5094 (154)*	11857 (254)*
Adenosine	3.10 (0.43)	446 (56)	2452 (395)	4650 (292)	10315 (286)
Inosine	3.38 (0.44)	526 (69)	2572 (386)	4532 (222)	10577 (312)
Hypoxanthine	3.49 (0.62)	581 (83)	2833 (468)	4361 (259)	10710 (329)
Uric acid	3.17 (0.45)	530 (70)	2257 (301)	4293 (261)	10467 (311)
IL-10 (pg/ml)	0 h	2 h	4 h	6 h	24 h
Medium	1.04 (0.07)	1.08 (0.09)	4.99 (0.66)	30.99 (3)	1966 (257)
ADP	0.95 (0.01)	1.22 (0.10)	4.92 (0.65)	34.75 (7)	2566 (143)*
AMP	0.98 (0.06)	1.39 (0.16)	4.65 (0.74)	32.00 (5)	1905 (227)
Adenosine	1.03 (0.06)	1.30 (0.13)	3.62 (0.25)	27.77 (4)	1437 (172)*
Inosine	0.93 (0.04)	1.09 (0.07)	3.93 (0.43)	28.19 (4)	1585 (220)*
Hypoxanthine	0.93 (0.09)	1.06 (0.05)	3.88 (0.39)	28.45 (5)	1517 (237)*
Uric acid	0.87 (0.03)	1.08 (0.04)	3.90 (0.37)	30.78 (5)	1608 (234)

* $p<0.05$ compared to cytokine release in the presence of medium (control condition).
[a] Data are shown as mean (SEM) in six subjects.

was used, indicating that the medium as such did not interfere with the degradation of ATP.

We then investigated the time-dependent effects of ATP on several inflammatory markers at different time-points after LPS-PHA stimulation. The chemokine IL-8 is responsible for inducing the directed migration of cells to a site of inflammation (chemotaxis) and has an important role in regulating the acute inflammatory response[27]. IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of many inflammatory proteins, including cytokines like TNF-α and chemokines and is among others produced by activated T cells[12]. Our study shows that TNF-α, IL-8 and IL-1β are released early and gradually after stimulation of blood by LPS-PHA, which is consistent with their role in acute inflammatory processes. IFN-γ release appeared to predominate at later time points, possibly reflecting its role in amplifying inflammatory processes. Finally, the predominant IL-10 release at the latest time point (24 h) may reflect its role in the resolution of inflammatory processes as a natural feedback mechanism. Our data show that ATP attenuates the stimulation-induced rise in concentrations of TNF-α, IFN-γ and IL-1β, but increases the stimulation-induced rise in IL-8 and IL-10 concentrations. In addition, our data indicate that ATP already exerts these anti-inflammatory effects within 2 h for TNF-α and IL-8 and within 4 h for IFN-γ, IL-1β and IL-10, and that these effects persist until 24 h for all cytokines. In contrast to the anti-inflammatory effects of ATP, there are numerous in vitro studies indicating that ATP can also exert immune-stimulating and proinflammatory effects. However, these pro-inflammatory effects occur at millimolar concentrations of ATP, thereby activating the P2X7 receptor[14, 15, 16, 28]. In our study, the concentration of ATP over the 24 h incubation period was in the micromolar range. Moreover, we earlier showed that the anti-inflammatory effects of ATP in this whole blood system are due to P2Y receptor activation and that activation of the P2X7 receptor was not involved[33].

It is well recognized that metabolites of ATP can also exert powerful modulatory effects on the immune system. We therefore tested the time-dependent effects of several metabolites of ATP on cytokine release in stimulated blood. Our data show that ADP has some similar effects as ATP but in contrast to ATP, ADP stimulated LPS-PHA-induced IL-1β production. Among all the tested metabolites, ADP is the only metabolite, which showed, just as ATP, a stimulatory effect on LPS-PHA-induced IL-10. This finding corroborates and expands on our recent report[33], which showed that the stimulatory effect of ATP on IL-10 release observed 24 h after stimulation was regulated via activation of the P2Y12 receptor, a receptor activated by ADP[11, 34].

The nucleoside adenosine is a well-known immunomodulatory agent and has been shown to inhibit deleterious immune-mediated processes, including the release of pro-inflammatory cytokines from different types of stimulated cells[4, 5, 17, 31, 34]. Surprisingly, in our study, adenosine showed no effect on LPS-PHA-induced TNF-α, IL-1β and IL-8 concentrations, but it inhibited IFN-γ and IL-10 release. Similar inhibitory effects of adenosine on IL-10 and IFN-γ release were shown earlier in different cell types by several groups[13, 29]. Inosine, which is formed by the breakdown of adenosine by adenosine deaminase, is also known to have potent

anti-inflammatory effects by inhibiting the release of pro-inflammatory cytokines and chemokines by different stimulated cell types[18, 23, 24]. In our stimulated blood model, inosine showed no effect on LPS-PHA-induced IFN-γ, IL-1β and IL-8 concentrations, but it showed an inhibitory effect on TNF-α and IL-10.

Very limited information is available on the pharmacological effects of hypoxanthine, the purine base that is cleaved off from inosine. In our study, hypoxanthine stimulated the rise in IL-1β concentrations and decreased the rise in IL-10 concentrations after LPS-PHA stimulation. The fact that hypoxanthine showed no effect on TNF-α release and an opposite effect on IL-10 release, compared to ATP, indicates that the release of hypoxanthine observed 24 h after LPS-PHA stimulation is not responsible for the anti-inflammatory effects of ATP observed 24 h after stimulation, when all ATP is metabolized.

Production of cytokines and chemokines is essential in immunity and involves the activation of several transcription factors, including NFκB[2]. In the resting state, NFκB is present in the cytoplasm of the cell through its tight association with the inhibitory protein IκB[21]. Upon cell stimulation, such as LPS, IκB is phosphorylated and degraded, followed by translocation of NFκB into the nucleus to regulate transcriptional activation of a host of cytokine genes and subsequent release of cytokines, including TNF-α, IL-6, IL-1β and IL-8[7, 22]. Our data show that NFκB is activated in a time-dependent fashion (at 2 until 24 h) after LPS-PHA stimulation in blood. Moreover, ATP was able to down-regulate the stimulation-induced NFκB activation at 2 h only, which indicates an early inhibitory effect of ATP on NFκB. The modest inhibitory effect of ATP on NFκB activation could suggest that other transcription factors or other intracellular pathways are also involved in the observed effects of ATP on cytokine production. Another possibility is that NFκB has already reached its maximum activation between 0 and 2 h after stimulation.

In conclusion, in the present study, we have further characterized the used LPS-PHA-stimulated blood model. In this model, ATP is slowly broken down and almost completely degraded after 6 h. During that time, ADP, AMP and hypoxanthine are formed, with a high release of hypoxanthine at 24 h. ATP inhibits the release of TNF-α, IFN-γ and IL-1β, but stimulates the release of IL-10 and IL-8 after LPS-PHA stimulation of blood. Our results demonstrate that ATP is able to modulate a variety of cytokines in stimulated blood; moreover, these immunomodulatory effects of ATP are rapid (at 2, 4 and 6 h after LPS-PHA stimulation) and persist until 24 h.

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Chapter 6

A comparison of ex vivo cytokine production and antioxidant capacity in whole blood after incubation with uric acid or monosodium urate crystals

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Abstract

Introduction: Deposition of uric acid in hyperuricemic patients can induce gout. Paradoxically, uric acid is also an important antioxidant. The aim of this study was to compare the effects of monosodium urate crystals (MSU) and uric acid crystals (UA) on inflammation and antioxidant capacity in the absence and presence of lipopolysaccharide (LPS) and phytoheamagglutinin (PHA) induced inflammation. Our study investigates this for the first time in whole blood instead of specific cultured cells.

Methods: Whole blood was incubated for 24h (37°C, 5% CO_2) with MSU crystals or UA crystals at doses of 0, 0.3, 4, and 10 mmol/L, with and without LPS/PHA. Multiplex measurement of 17 cytokines, and determination of soluble uric acid concentration and antioxidant capacity were done in cell-free supernatant.

Results: The soluble uric acid concentration and antioxidant capacity of cell-free supernatants was higher for UA compared to MSU crystals, but did not increase further when incubations above the solubility level of uric acid of ~420 µmol/L were employed. MSU crystals dose-dependently induced cytokine production to levels which were up to 300 times higher than cytokine production induced by UA crystals. In LPS/PHA-stimulated blood, cytokine release was inhibited by co-incubation with either MSU crystals or UA crystals.

Conclusion: In unstimulated whole blood, MSU crystals have a marked proinflammatory effect, which is not seen for UA crystals. The anti-inflammatory effect of both MSU and UA in LPS/PHA-stimulated blood may be explained by the presence of soluble uric acid and the resulting increase in the antioxidant capacity of the blood.

Introduction

Humans and higher primates have considerably higher plasma uric acid concentrations than most other species because they lack the enzyme uricase. This is probably the result of parallel, independent mutations of the gene coding for uricase in two lineages leading to higher primates in the Miocene (5-23 million years ago) [1]. As a consequence, uric acid is the enzymatic end product of purine metabolism in humans, instead of the more soluble and readily excreted allantoin in other mammals [2]. The fact that these mutations were able to maintain themselves, strongly suggests that there must have been a selection advantage to having higher plasma uric acid concentrations. Several hypotheses have been put forward. The first and most quoted hypothesis involves the important role uric acid plays as an antioxidant in plasma [3]. Proctor [4] and later Ames [3] suggested that the uricase mutation may have benefited survival because it maintained adequate plasma antioxidant activity after the loss of ascorbate synthesis due to an earlier mutation [5].

Another hypothesis focuses on the function of uric acid in the innate immune system. Specifically, uric acid may aid in the immune recognition of dying cells [6], the immune rejection of tumor cells [7], and help activate the inflammasome responsible for interleukin-1-beta (IL-1ß) release[8, 9]. More recently, uric acid release from cells in the area directly surrounding a site of vaccine injection has been found to enhance the effectiveness of the vaccine[(10), reviewed in (11)]. A third hypothesis argues that a higher uric acid concentration may have conferred a survival advantage in early primates because it helped maintaining blood pressure during periods of dietary change and environmental stress[12]. In the current study we focussed on the antioxidant properties of uric acid in relation to their anti- or pro-inflammatory actions.

Although exerting mainly beneficial effects at normal plasma concentrations of 175-355 µmol/L, uric acid can give rise to crystallization at concentrations beyond 420 µmol/L. Deposits of monosodium urate (MSU) crystals ($Na_5C_5H_{30}N_4O_3 \cdot H_2O$) can develop after prolonged periods of hyperuricemia and are the causative agent for the painful inflammation of the joints that is typical for gout[13, 14]. MSU crystals are recognized by Toll-like receptors (TLR) 2 and 4[15] and during a gout attack this triggers local synovial cells, macrophages and neutrophils to produce the classical pro-inflammatory cytokines IL-6, IL-8, IL-1ß, and TNF-α[16-19]. Injection of MSU crystals into the joints of animals induced the typical symptoms of gout. The response was greatly diminished when neutrophils were depleted[20], stressing the importance of this cell type. Neutrophils release reactive oxygen species (ROS) such as superoxide, when exposed to MSU crystals[21]. The resulting oxidative damage acts as a priming agent for inflammation through activation of transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1 (AP-1)[22]. NF-κB in its turn enhances the expression of inflammatory genes, encoding for instance TNF-α, IL-6 and IL-8[23].

The rationale for the current study was based on the dual role of uric acid: on the one hand strongly contributing to the total antioxidant capacity of plasma and, on the other hand, its pro-inflammatory functions, partly caused by the superoxide released from MSU crystal-stimulated neutrophils. We hypothesized that uric acid in solution would exert beneficial effects on total antioxidant capacity. The resulting reduction in free radical formation would then diminish the trigger for inflammation. As a result, uric acid in solution would be expected to exhibit predominantly anti-inflammatory effects. MSU crystals, however, are expected to exhibit predominantly pro-inflammatory effects through their strong direct activation of neutrophils and macrophages. At high micromolar concentrations, uric acid cannot be dissolved, and undissolved uric acid particles (further referred to as UA crystals) will be present next to uric acid in solution. Differences in physical properties of crystals are known to lead to differences in the intensity of the inflammatory response[24]. We hypothesized that MSU crystals would exert a stronger pro-

inflammatory response than UA crystals. To investigate this, we incubated human blood with different concentrations of UA crystals and MSU crystals, and determined the antioxidant capacity and the inflammatory activity using a panel of 17 pro-and anti-inflammatory cytokines.

Material and methods

Chemicals

Purified phytohaemagglutinin (PHA) HA16 was obtained from Remel Europe Ltd, UK. Lipopolysaccharide (LPS), NaOH, uric acid and 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma-Aldrich, St. Louis, MO, USA. 2,2'-Azobis (2-Amidinopropane) dihydrochloride (ABAB) was obtained from Polysciences Inc, Warrington, PA, USA. $Na_2HPO_4*2H_2O$ and $NaH_2PO_4*H_2O$ were obtained from Merck, Darmstadt, Germany. RPMI 1640 medium (+L-glutamine) was obtained from Gibco, UK.

MSU crystal preparation

MSU crystals were prepared by heating a 30 mmol/L uric acid solution in 1 M NaOH to 70°C while stirring. The pH of the warm solution was lowered to pH 5.8 by adding HCl. The solution was then incubated overnight at room temperature while stirring slowly. The resulting suspension was subsequently centrifuged at 3000 x g for 10 min and the supernatant discarded. Following resuspension in RPMI 1640 medium, working suspensions were made containing approximately 0.4, 5.2, and 13.3 mmol/L MSU. The crystals exhibited a striking needle-like shape and measured between 20 and 80 μm in length, when checked by microscope (figure 1).

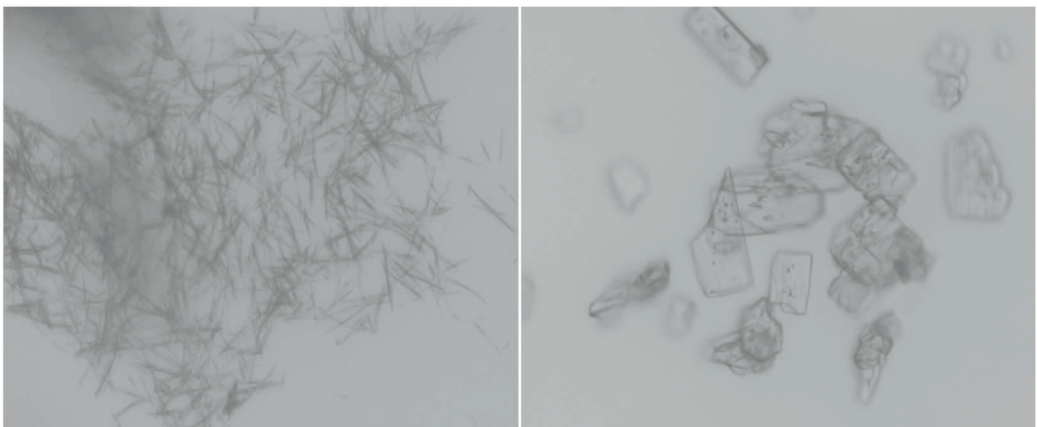

Figure 1: MSU crystals (left) and UA crystals (right) at 40x magnification.

Uric acid preparation

A 106.7 mmol/L uric acid suspension was made in water by sonification for 10 min at room temperature. This concentration far exceeds the solubility of uric acid in water. Therefore, this preparation also contained undissolved particles, further referred to as 'UA crystals'. The shape of these crystals was very different from the MSU crystals (figure 1). Working solutions were made by dilution with RPMI 1640 medium to concentrations that yield the desired concentration of UA crystals upon further dilution with blood in the whole blood assay.

Whole blood assay

The whole blood assay was conducted according to Swennen et al.[25]. Blood was collected from five healthy volunteers (three women and two men) in heparin-containing vacutainer tubes (Vacutainer, Becton-Dickinson, 170 i.u., Franklin Lakes, NJ, USA), immediately inverted several times and placed on ice. The incubation was started within 30 minutes after blood collection. Whole blood (250 µL per well) was added to 24-well sterile plates and diluted 1:4 with RPMI 1640 medium. Working solutions of UA crystals or MSU crystals were added to the wells to attain final doses of 0 ('control'), 0.3 ('low'), 4 ('mid'), and 10 ('high') mmol per L. While the MSU crystals remained intact and did not dissolve, the UA crystals dissolved completely at the lowest concentration, but incompletely at the middle and high concentrations. Incubation was conducted in the presence ('stimulated samples') or absence ('unstimulated samples') of LPS and PHA at final concentrations of 10 and 1 µg/mL. The plates were then incubated at 5% CO_2, 37°C for 24h. Following resuspension and centrifugation (9,500 x g, 8 min at 4°C) of the contents of the well, cell free supernatants were collected, aliquoted and stored at -80°C.

Cytokine Assay

Human cytokines were measured according to the manufacturer's instruction by a multiplex immunoassay, containing dyed microspheres conjugated with a monoclonal antibody specific for a target protein (Bioplex, 17-plex multiplex kit Human Grp I cytokines, Bio-rad Laboratories Inc., Hercules, CA, USA). We measured the cytokines IL-1β (IL1F2), IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL- 12 (p70), IL-13, IL-17, tumor necrosis factor-alfa (TNF-α), and interferon-gamma (IFN-γ), and the chemokines CXCL8 (IL-8), granulocyte colony stimulating factor (G-CSF), granulocyte–monocyte colony stimulating factor (GM-CSF), CCL2 (monocyte chemoattractive protein [MCP]-1), CCL4 (macrophage inflammatory protein [MIP]-1β). Briefly, supernatant samples were diluted 1:10 (stimulated samples) or 1:4 (unstimulated samples), and incubated with antibody-coupled beads. Complexes were washed, then incubated with biotinylated detection antibody and, finally, with streptavidin–phycoerythrin prior to assessing cytokine concentration titers. Concentrated recombinant human cytokine was diluted up to 262,000 times (with the maximum concentration for each cytokine as reported by the vendor (BioRad Inc.)) to establish standard curves with maximum sensitivity and dynamic range. Cytokine le-

vels were determined using a Bio-Plex array reader (Luminex®100™ IS, Luminex B.V., Oosterhout, The Netherlands). Data analysis was done with Bio-Plex Manager software (Bio-Rad Laboratories) with 5-parametric logistic curve fitting. The derived equation was then used to calculate the concentration of cytokines in plasma samples.

Uric acid measurement

The concentration of uric acid was measured by HPLC, as described by Coolen et al. with minor modifications[26]. Following thawing on ice, 100 µL sample was added to 100 µL 8% PCA. Following vortex-mixing and centrifugation at 12,000 g (5 min, 4°C), 2M K_2CO_3 in 6 M KOH was added to 50 µL sample to neutralize the pH. Finally, the samples were diluted 1:1 with mobile phase. Uric acid was quantified at 254 nm following separation over a 3 µm particle size Allsphere C18 reverse phase column (150 x 4.6 mm i.d.; Grace Alltech, Breda, The Netherlands).

TEAC assay

The Trolox-equivalent antioxidant capacity (TEAC), which reflects the ability of lipid and aqueous antioxidants to scavenge the stable ABTS radical ($ABTS^{\cdot+}$), was determined in cell-free supernatants as described previously[27-29]. To generate $ABTS^{\cdot+}$, 10 mg of ABTS and 44 mg of ABAB were dissolved in 70 mL of a 145 mM sodium phosphate buffer of pH 7.4. The solution was heated to 70°C until the absorption at 734 nm wavelength reached 0.7 ± 0.02, and then quickly cooled on ice. To determine the TEAC, 950 µL of the stable $ABTS^{\cdot+}$ solution was preincubated for 1 minute at 37°C, and then 50 µL of cell-free supernatant was added and incubated at 37°C for 5 minutes exactly. Absorption was measured at 734 nm using a Cary50® scan spectrophotometer (Varian Inc., Palo Alto, CA, USA). TEAC is reported as micromoles of Trolox equivalent (a water-soluble tocopherol analogue). Dilution was increased to 100 times (10 µL of sample added to 990 µL stable $ABTS^{\cdot+}$ radical solution) when the absorbance fell below 0.2.

Statistical analysis

The cytokine and chemokine concentrations measured in the control samples were subtracted from the concentrations measured in the presence of UA crystals or MSU crystals, and these changes from the control incubation were plotted (Figure 2). A paired samples t-test was done to determine whether the changes were statistically significant, and to determine whether the changes were different for MSU crystals and UA crystals. Values are presented as means ± SEM. $P<0.05$ was regarded as statistically significant.

Results

Uric acid concentration

Cell-free supernatants were collected after whole blood was exposed ex vivo to control, low, mid, or high doses of either UA crystals or MSU crystals. The measured concentrations of uric acid in solution in these supernatants increased with increasing UA crystal doses added (Table 1). While concentrations of uric acid, as expected, approached the solubility level of uric acid in water (approximately 420 µmol/L[30]) for the mid and high doses, the concentration in the low dose was six times lower than anticipated: 47 µmol/L where 300 µmol/L was expected. When increasing doses of MSU crystals were added, the uric acid concentration did not increase beyond ~100 µmol/L. Stimulation of the blood with LPS/PHA did not affect the measured uric acid concentrations (Table 1).

TABLE 1. URIC ACID CONCENTRATIONS (µMOL/L) IN CELL-FREE SUPERNATANTS OF WHOLE BLOOD INCUBATED FOR 24 HOURS IN THE PRESENCE OF 0 (CONTROL), 0.3 (LOW), 4 (MID), OR 10 MMOL/L (HIGH) DOSES OF EITHER MSU CRYSTALS OR UA CRYSTALS. INCUBATIONS WERE CARRIED OUT IN THE PRESENCE OR ABSENCE OF LPS/PHA. VALUES ARE MEANS ± SEM FOR N=5.

	Uric acid concentration (µmol/L)							
	Control		Low		Mid		High	
Crystal dose added	0		300		4,000		10,000	
UA crystals								
- LPS/PHA	10	±3	47	±3	300	±13	394	±48
+ LPS/PHA	11	±3	47	±5	342	±23	445	±29
MSU crystals								
- LPS/PHA	10	±3	29	±2	98	±2	102	±13
+ LPS/PHA	8	±1	27	±1	106	±4	104	±16

TABLE 2. TROLOX EQUIVALENT ANTIOXIDANT CAPACITY (TEAC) IN CELL-FREE SUPERNATANTS OF WHOLE BLOOD INCUBATED FOR 24 HOURS IN THE PRESENCE OF 0 (CONTROL), 0.3 (LOW), 4 (MID), OR 10 MMOL/L (HIGH) DOSES OF EITHER MSU CRYSTALS OR UA CRYSTALS. INCUBATIONS WERE CARRIED OUT IN THE PRESENCE OR ABSENCE OF LPS/PHA. VALUES ARE MEANS ± SEM FOR N=5.

	Trolox equivalents (µmol/L)							
	Control		Low		Mid		High	
UA crystals								
- LPS/PHA	1648	±51	1441	±196	3581	±69	4471	±343
+ LPS/PHA	1603	±45	1430	±179	3522	±126	4332	±323
MSU crystals								
- LPS/PHA	1519	±331	2064	±80	2562	±25	2575	±90
+ LPS/PHA	1484	±320	1992	±106	2615	±21	2570	±29

Antioxidant capacity

The antioxidant capacity of the control incubation was ~1500 µmol/L Trolox equivalents (Table 2). As expected, the total antioxidant capacity increased when blood was incubated with increasing UA or MSU crystal doses. For UA crystals, TEAC increased up to ~4400 µmol/L, whereas for MSU crystals it increased only

up to ~2500 μmol/L. The increase in antioxidant capacity leveled off at the mid dose. Stimulation with LPS/PHA did not influence the antioxidant capacity.

Inflammatory effects

Control incubations. In the control condition for this experiment (absence of both LPS/PHA and UA crystals or MSU crystals), cytokine concentrations measured in the cell-free supernatants were very low (Table 3). Stimulation of blood with LPS/PHA for 24 hours induced production of all 17 cytokines, although to a varying degree (Table 3). Some cytokines were present at very low concentrations (<100 pg/mL: IL-4, IL-5, IL-7, and IL-13), some at medium concentrations (100-1000 pg/mL: GM-CSF, IL-2, IL-12, IL-17), while some were abundantly present after exposure to LPS/PHA (>1000 pg/mL: TNF-α, IFN-γ, G-CSF, IL-1β, IL-6, IL-8, IL10, MIP-1b, and MCP-1). The concentrations of IL-6 and IL-8 were beyond the measurable range after blood was incubated with LPS/PHA.

TABLE 3. CYTOKINE CONCENTRATIONS (PG/ML) IN CELL-FREE SUPERNATANT OF WHOLE BLOOD INCUBATED FOR 24 HOURS AT 37°C AND 5% CO_2 IN THE PRESENCE OR ABSENCE OF LPS/PHA. VALUES ARE MEANS ± SEM FOR N=5.

Cytokine	–LPS/PHA		+ LPS/PHA	
TNF-α	1.2	±0.2	16,495	±4,271
IFN-γ	3.3	±1.7	29,948	±6,734
G-CSF	0.2	±0.1	2,587	±718
GM-CSF	2.4	±0.6	448	±84
IL-1β	1.5	±0.6	17,369	±7,893
IL-2	3.9	±1.5	225	±34
IL-4	0.2	±0.1	43	±5
IL-5	0.7	±0.0	7	±3
IL-6	8.5	±3.0	(OOR)[1]	
IL-7	3.1	±0.5	6	±1
IL-8	16.8	±5.3	(OOR)[2]	
IL-10	1.0	±0.3	2,442	±615
IL-12	17.5	±8.9	367	±162
IL-13	0.6	±0.1	66	±19
IL-17	12.0	±3.2	989	±130
MIP-1b	(OOR)[3]		(OOR)[3]	
MCP-1	16.3	±3.6	1,385	±684

[1] Out of range, concentration ≥33,000 pg/ml.
[2] Out of range, concentration ≥ 30,000 pg/ml.
[3] Out of range, concentration ≥ 27,000 pg/ml.

Incubations with UA or MSU crystals. Figure 2 shows the changes in cytokine concentrations for blood incubated with UA crystals or MSU crystals relative to control blood. Some or all values for IL-1β, MIP-1b, IL-6 and MCP-1 were above the range that could be accurately measured. MIP-1b is therefore not included in Figure 2.

In unstimulated blood (i.e. without LPS/PHA), incubation with MSU crystals resulted in a strong induction of most cytokines (Figure 2, left panels). For all cytokines except IFN-γ, IL-5, IL-7, IL-12, and MCP-1, the increase in cytokine production compared to the control incubation was statistically significant for at least one dose of MSU crystals (Figure 2, left panels, white bars, indicated with #). In contrast, incubation with UA crystals resulted in a much weaker induction of cytokine production, even though the induction was still statistically significant for TNF-α, IFN-γ, GM-CSF, IL-1β, IL-2, IL-4, IL-8, IL-10, and IL-17 (Figure 2, left panels, grey bars, indicated with #). When compared with similar doses of UA crystals, MSU crystals induced a significantly higher production of TNF-α, G-CSF, GM-CSF, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, and IL-17 (figure 2, left panels, white bars, indicated with *).

In blood stimulated with LPS/PHA, incubation with UA crystals tended to lower cytokine concentrations compared to the control incubation with LPS/PHA only. A statistically significant reduction was observed for the cytokines IFN-γ, IL-5, and IL-10 (Figure 2, right panels, gray bars, indicated with #). MSU crystals together with LPS/PHA also tended to inhibit cytokine production (Figure 2, right panels, white bars, indicated with #). This reduction was statistically significant for the cytokines TNF-α, IFN-γ, IL-5, IL-7, IL-10, and IL-13. For IL-7 and IL-10, the inhibition by MSU crystals was significantly stronger than that by UA crystals, as indicated by * in the right panels of figure 2.

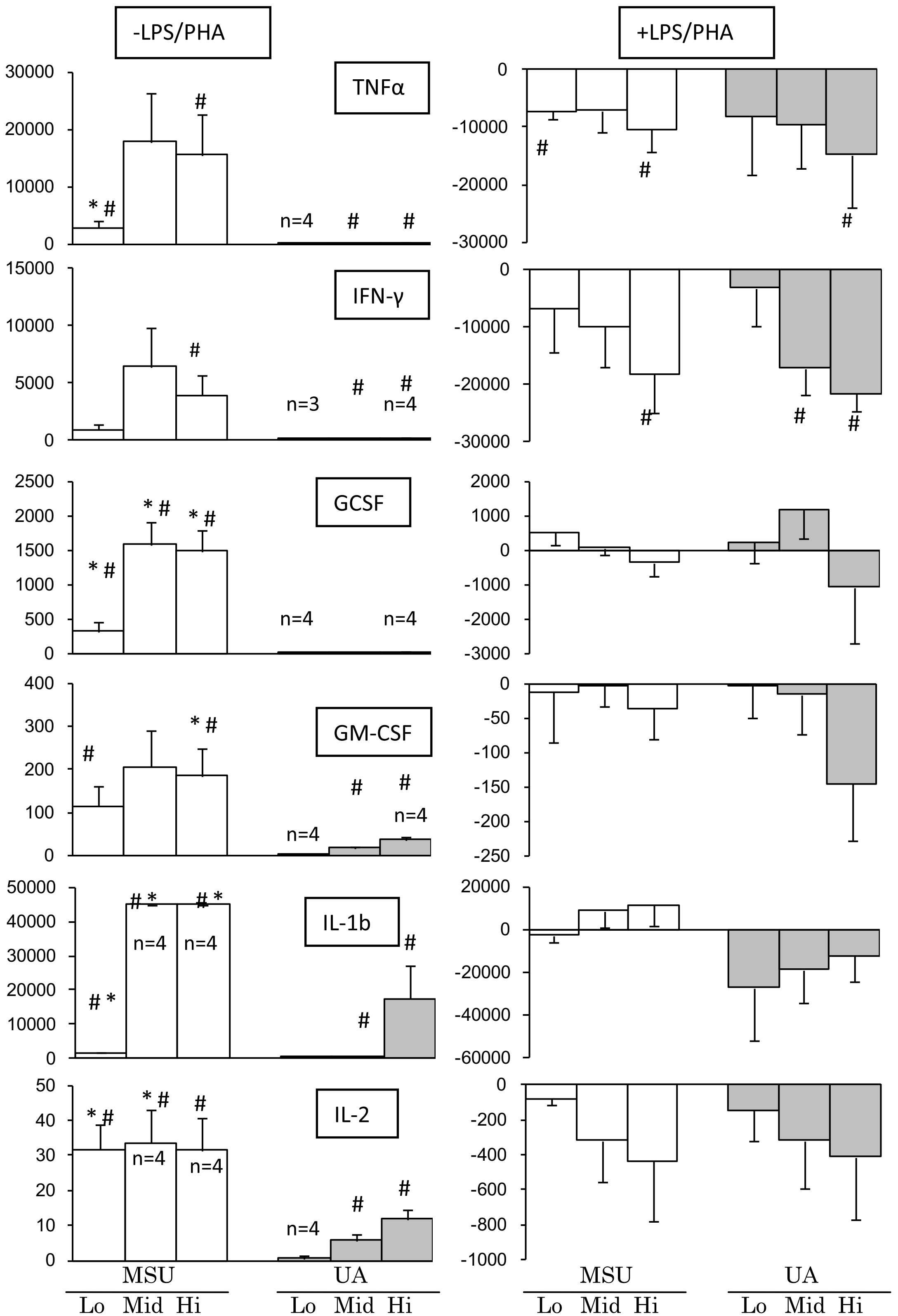

Figure 2 (continued on next page)

-LPS/PHA

+LPS/PHA

IL-4

IL-5

IL-6

all out of range

IL-7

IL-8

IL-10

MSU

Lo Mid Hi

UA

Lo Mid Hi

MSU

Lo Mid Hi

UA

Lo Mid Hi

Figure 2 (continued on next page)

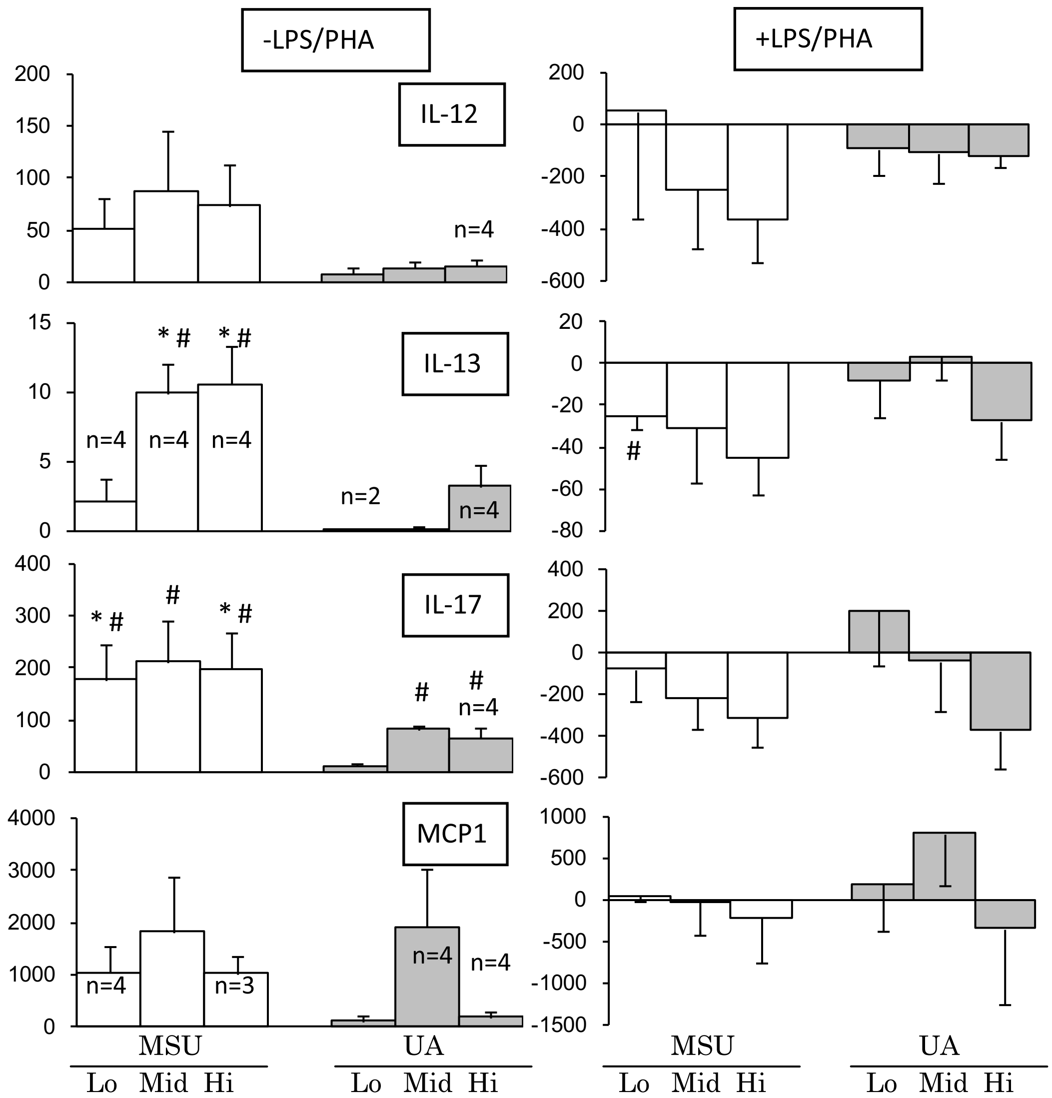

Figure 2: Changes in concentration (pg/mL) of cytokines and chemokines after incubation of whole blood for 24 h in the presence of Low, Mid, and High doses of MSU crystals (white bars) or UA crystals (grey bars), compared to controls without crystals. Incubations were carried out in the absence (-LPS/PHA; left panels) or presence (+LPS/PHA; right panels) of LPS/PHA as an inflammatory stimulus. Values are means ± SEM for n=5. When data were missing, the number of samples included in the analysis is indicated in the figure.
* Statistically significant difference between incubation with MSU and UA crystals ($p<0.05$).
# Statistically significantly different from 0 ($p<0.05$).

Discussion

Our aim was to investigate whether ex vivo incubation of whole blood with varying doses of UA crystals or MSU crystals would modify the production of cytokines and chemokines, both in native blood and in LPS/PHA-stimulated blood ex vivo. At the same time, we measured the antioxidant capacity and uric acid concentrations in the incubated blood.

Several diseases are caused by depositions of crystals, mainly in articular cartilage tissues in the joints. The best-known condition is gouty arthritis in which MSU crystals are deposited within the joints. A similar disease is calcium pyrophosphate dehydrate disease (commonly known as pseudogout), which is caused by accumulations of crystals of calcium pyrophosphate dehydrate. In the United States, around 50% of the population over 85 years of age are affected. A third example of crystal-induced diseases is osteoarthritis which is linked to carbonate-apatite crystals. The symptoms of all include red, tender and swollen joints. Since gouty arthritis, has by far the highest prevalence, we focussed our investigation on monosodium urate crystals.

Inflammatory effects of UA crystals and MSU crystals

Results show that in unstimulated blood, MSU crystals induced nearly all cytokines up to concentrations that were 300 times higher compared to UA crystals. This confirms that MSU crystals are strong inducers of inflammatory responses. Moreover, the effect was dose-dependent for most cytokines. In contrast, in LPS/PHA-stimulated blood, both MSU crystals and UA crystals exhibited an anti-inflammatory effect, as they lowered the concentrations of cytokines that were induced by LPS/PHA.

We observed a very low spontaneous production of cytokines in non-stimulated blood, corroborating earlier whole blood studies[31]. The strong increase in cytokine production upon stimulation with LPS/PHA was also expected and is attributable to the combined activation of the T-lymphocyte by PHA, and the TLR 4 pathway by LPS, which activates the B-lymphocytes that are present in circulation[32]. IL-12, for instance, has been shown to be normally only produced when TLR ligands like LPS are present[33]. In a set-up similar to ours, but using a 10,000 times weaker LPS stimulus, Eriksson *et al.* found an only 8 times lower TNF-α concentration, which indicates that LPS can evoke inflammation at much lower concentrations[31].

As hypothesized, in the absence of LPS/PHA MSU crystals strongly induced the production of all cytokines except IL-5 (figure 2, left panels). There was a particularly strong increase in TNF-α, IL-1ß, IL-6, IL-8, and MIP-1ß concentrations. These cytokines were also among the strongest responders in previous studies[16, 17, 34, 35]. The opposite was true in blood stimulated with LPS/PHA: here, MSU crystals did not enhance production of cytokines, but even inhibited the production of some cytokines. This result was not in accordance with our hypothesis. Taken together, this indicates that MSU crystals as such act pro-inflammatory, but do not have this effect under challenged (+LPS/PHA) conditions.

Other studies showed that MSU crystals stimulate the synthesis and release of IL-1ß and the neutrophil chemoattractant IL-8 from neutrophils[36, 37]. Both IL-1ß and IL-8 have been shown to amplify the acute inflammatory reaction of gout: on the one hand, IL-8 is abundant in synovial fluids of patients with gout[18] and on the other hand, treatment using the IL-1 receptor antagonist anakinra has been

shown to be effective in the control of gout[38]. A strong induction of MIP-1β by MSU was reported to occur in macrophages, which was linked to activation of the Erk1/2 signalling cascade[39]. Besides neutrophils and macrophages, eosinophils have recently also been found to be attracted to MSU crystals[40]. In that study, eosinophils were reported to be activated through P2Y2 or P2X7 receptors: IL-6 and IL-8 were reported to be produced when eosinohils were incubated with MSU crystals (range 0-0.1 mg/ml; equivalent to 0-1.5 mmol/L), but not when incubated with soluble uric acid. That study also reported up-regulation of the cytokines IL-1β, IL-10, IL-17, FGF, G-CSF, GM-CSF, IFN-γ, MCP1, MIP1α and β, TNF-α and TGF-β by MSU crystals, mimicking our results[40].

Instead of a specific subset of blood immune cells, some authors used isolated PBMCs (consisting of lymphocytes, monocytes and macrophages together) to investigate the effects of incubation with MSU crystals at doses up to 3.6 mmol/L[41]. The increase in TNF-α in this study was weak, as opposed to ours, which may be due to the presence of factors whole blood that may be needed for MSU crystals to activate the inflammasome[9]. Whole blood cultures have been shown to be as good as PBMC cultures for studying inflammatory reactions, and they provide two major advantages: 1, all cellular components of peripheral blood are present in their natural proportions and 2, preactivation or desensitation of immune cells, often brought about by separating PBMCs on density gradients, is less likely to occur[42].

Next to incubations with components of blood, animal models measuring crystal-induced neutrophil infiltration into the peritoneal cavity have been established. Injection of an MSU crystal dose of 1 mg of crystals/mouse, resulted in increased neutrophil infiltration, but decreased macrophage infiltration[43]. The neutrophil infiltration by MSU crystals was found to be dependent on the inflammasome to produce IL-1β, as was earlier also found to be true for inorganic particles such as asbestos fibres, silica particles and alum crystals[44, 45].

In contrast to MSU crystals, UA crystals were only weak inducers of inflammation, with statistically significant induction of the pro-inflammatory cytokines TNF-α, GM-CSF, IFN-γ, IL-8, and IL-17 as well as the anti-inflammatory cytokines IL-10 and IL-4. Of note, this occurred only at the highest UA crystal doses and, when compared with MSU crystals, the increase was less pronounced. This finding would suggest that the physical properties of crystals help determine the intensity of the resulting inflammatory response. Shi *et al.* already recognized that the crystalline form of MSU crystals acts as a ‘danger-signal’[6]. MSU crystals can destabilize phagosomes leading to phagosome rupture and, via activation of the NALP-3 inflammasome, to the conversion of pro-IL-1β to its active form IL-1β, mainly by caspase-1[44]. Other crystals such as calcium pyrophosphate dihydrate are also known to be able to activate this pathway[24].

The question remains why UA crystals and MSU crystals, in the presence of LPS/PHA, both failed to increase the cytokine and chemokine production. A previous study in which isolated PBMCs were stimulated with MSU crystals in com-

bination with LPS, showed synergy of MSU and LPS on the production of IL-1β and, to a smaller extent, TNF-α, but not IL-6[41]. In that study, however, 1000 times lower LPS concentrations were used, which leaves open the possibility that in our set-up, the cytokine production is already at such a high level that it can not be increased further by MSU crystals. Future studies will have to shed more light on this issue.

Uric acid concentrations and anti-oxidant capacity

We were also interested in the effect of the MSU crystals on antioxidant capacity of the whole blood. Since uric acid is a strong contributor of antioxidant capacity of blood, the uric acid concentration was of particular interest. The results of the soluble uric acid concentration measurements indicated that both MSU crystal and UA crystal suspensions contained non-crystalline uric acid in solution. As hypothesized, this may explain their anti-inflammatory effects, not only for the UA crystal incubation, but also for the MSU crystal incubation.

An increase in antioxidant capacity of the supernatant samples was expected with increasing UA or MSU crystal dose[3]. Our results indeed confirm this. However, there was a discrepancy between measured uric acid concentrations and doses added for incubation. This can largely be explained by the limited solubility of uric acid. At the Mid and High dose incubations, doses added were above the solubility limit of uric acid of 420 μmol/L[46]. As expected, we found no concentrations of soluble uric acid above this limit. At the low UA dose of 0.3 mmol/L, however, measured concentrations were lower than expected, and we have no good explanation for this.

Our results confirm that the ex vivo whole blood model is useful to monitor antioxidant capacity, uric acid concentration and cytokine production. Our results show for the first time in what way monosodium urate crystals interact with whole blood. MSU crystals showed a pro-inflammatory effect in blood ex vivo which was markedly stronger than for UA crystals, as indicated by increased cytokine concentrations. In contrast, in blood samples stimulated by LPS/PHA, both MSU crystals and UA crystals lowered the concentrations of cytokines and chemokines. A possible explanation for this may be that the already high LPS/PHA-induced cytokine production can not be increased further, while the increased antioxidant capacity due to increased concentrations of soluble uric acid, results in an anti-inflammatory effect.

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Chapter 7

General discussion

General discussion

The main objectives of the research presented in this thesis were to:

i) establish and validate a method for measuring ATP and metabolites in biological samples;
ii) investigate the effect of single or repeated oral ATP administration on circulating concentrations of ATP and metabolites;
iii) investigate the effects on inflammatory markers and antioxidant capacity of ATP and its metabolites, including uric acid and monosodium urate (MSU) crystals in an ex vivo whole blood model.

In this chapter the most important findings and implications will be discussed. Finally, general conclusions and directions for future research will be suggested.

Optimization of a chromatography method for measuring ATP and metabolites in biological samples

In our human experiments (chapters 3 and 4), one of the goals was to determine the timing of release of ATP from enteric coated pellets that were administered orally. In order to accurately achieve this goal, blood samples were taken, yielding up to 23 samples per volunteer per day.

The use of direct 'bed-side' measurement of ATP (using for instance luminometry) was considered, since this could reduce the possibility of unwanted degradation of ATP and metabolites by for instance freezing and thawing of the samples. Such a method, however, would allow us to measure ATP only, since no luminometry method is available to measure the metabolites.

Therefore, we optimized a chromatography method and its sample processing to enable the simultaneous measurement of ATP and several metabolites in biological samples in one run (**Chapter 2**). The method was shown to be both simple, low-cost, and robust. Furthermore, the possibility of delayed measurement (after stabilizing and freezing the samples immediately following collection) is an important asset of chromatography that made it ideal for our experiments. Degradation of ATP in the samples was prevented by using EDTA blood collection tubes and by mixing the blood with perchloric acid directly after blood collection. EDTA chelates calcium which makes calcium unavailable as a cofactor for the enzymes that break down ATP. While these necessary stabilization steps made the sample preparation quite elaborate and required careful calibration, the average measurement time of one sample in the HPLC of approximately 20 minutes including maintenance and calibration time, was still very short.

Oral administration of ATP.

In order to explore the bioavailability and effects of oral administration of ATP, we performed two human experiments. In the first experiment (**chapter 3**), a single dose of a newly developed enteric coated oral mode of ATP administration was compared to an established mode of administration directly into the upper small intestine via a naso-intestinal tube. It was demonstrated that both administration methods gave similar results: an increase in uric acid in blood, but no increase in the levels of ATP or other metabolites. In the second experiment (**chapter 4**), the enteric coated mode of oral administration was given for four weeks. Similar to the findings of the first experiment, increased concentrations of uric acid, but no change of ATP or other metabolites concentrations, was observed. Before discussing the results of these experiments, I will first discuss purines in terms of extracellular signaling functions and their potential role as therapeutics, highlighting research findings on intravenous and oral ATP administration. Oral ATP administration will be discussed in general following the discussion of both individual studies.

ATP: state of the art

One of the first facts most students learn in biology class is that there is a universal energy currency used by all living cells which is called ATP. ATP is perhaps the most produced and consumed molecule in the human body, with a human using up its body weight of ATP over the course of one day[1]. Thus, ATP is a very important player in the biological field, driving all reactions that allow cells to function and make life possible.

Extracellular signaling functions of ATP

Less commonly known, but demonstrated beyond doubt by discoveries over the last 50 years, is that ATP is a critical signaling molecule that allows cells throughout the body to communicate with each other. For this function, ATP acts extracellularly in concentrations that are kept several thousand-folds lower than those inside the cell. In the 1970s, ATP was identified to act as a neurotransmitter on P2X and P2Y receptors[2]. In the 1990s, with the aid of molecular tools, ATP receptors were identified on many different cell types[3]. P2X receptors were found to be ion channels that allow sodium and calcium ions to enter cells upon activation by ATP. P2Y receptors are G-protein coupled receptors, which start a cascade of intracellular signaling after binding of ATP. Since ATP is such an ubiquitous molecule, its signaling functions have a broad influence on physiology: from muscle contraction, inflammation, vision, hearing, taste, nociception to bone building. The distribution of different receptor subtypes on different tissues has led to a search by laboratories worldwide to develop highly selective drugs, such as clopidogrel, a selective $P2Y_{12}$ receptor antagonist acting to prevent coagulation by ATP in blood [4]. The fact that clopidrogrel has been the 2nd top-selling drug since 2007 (sales of US$6.6 billion in 2009), indicates the large market potential for P2-receptor-based

therapeutics. Other drugs are under development, especially aided by the discovery of the molecular structure of several other P2 receptors.

Earlier studies on intravenous and oral ATP administration

Next to these drugs that target specific receptors, the administration of ATP itself has been investigated in randomized clinical trials in patients with cancer and rheumatoid arthritis[5-13]. These studies showed increased concentrations of ATP in the erythrocytes following intravenous administration, together with favourable clinical effects including inhibition of involuntary weight loss and pain, and improved life expectancies[5, 7, 9, 14, 15]. Although Beijer et al. established that low-dose ATP infusions (up to 50 μg/kg/min) can be safely administered in the home setting[13], several drawbacks adhere to the intravenous administration of ATP. These include difficulties in logistics and the cost of providing a continuous infusion in a home care setting. Moreover, it can be a burden for patients to undergo 24-h infusions, and i.v. access can be difficult to obtain.

These drawbacks do not apply to oral administration of ATP, which also allows more frequent and flexible dosage regimens to be used. The oral route, however, has not been investigated extensively so far. Only relatively low dosages up to 225 mg ATP per day have been clinically evaluated in human subjects, with outcome measures varying per study (blood levels of ATP, analgesic use, reported disability, etc.)[16-18]. While some beneficial effects were observed in these studies, they could not be directly attributed to ATP due to limitations in the design of the studies and a lack of ATP measurements in the circulation. These findings provided the rationale to further evaluate whether ATP can be effectively administered orally.

Discussion of the results of human experiments

Given a) the positive effects of intravenous ATP administration in cancer and rheumatoid arthritis patients, b) the wish for an easier method of administration, and c) the limited number of oral ATP administration studies, we designed two studies to thoroughly investigate the oral administration of ATP.

Direct administration vs. enteric coating

The aims of the first study (**Chapter 3**) were:

- i) to evaluate the suitability of enteric coated pellets as a means to orally administer ATP;
- ii) to establish whether the bioavailability of ATP changes when different parts of the small intestine are targeted, and;
- iii) to compare the results with earlier studies on oral ATP administration.

To achieve the first aim, the newly developed mode of ATP delivery was compared to an existing method which delivers the same amount of ATP directly into the small intestine. This means we compared enteric coated microparticles or 'pellets' (Ø 1 mm) to administration by duodenal tube. We hypothesized that both

modes of administration would have similar effects. This was indeed the case, although after administration of single doses of 250, 1250 or 5000 mg ATP, there was no effect on any of the measured ATP or metabolite (ADP, AMP, adenosine, adenine, inosine, and hypoxanthine) concentrations in blood, except for uric acid. Since uric acid is the final metabolite of human catabolism of ATP, the source of this increase appears to be directly traceable to the administration of ATP.

To achieve the second aim, the enteric coating of the pellets was adapted to release pellets in different areas of the small intestine. Based on the observation that the distal part of the small intestine in mice contained the smallest number of ATPases[19], we hypothesized that release of ATP in exactly this part of the small intestine would have beneficial effects on the stability of ATP and hence on its uptake. Instead, results showed that the increase in uric acid concentration was most pronounced for pellets with ATP delivery in the proximal small intestine. A potential explanation could be differences between humans and mice in intestinal makeup of the small intestine. The marker that we used to check for proper release of the contents of the pellets, lithium, however, was also absorbed to a very limited extent in the distal small intestine. This would suggest that the distal release pellets may have failed to release their contents in the small intestine, instead releasing them in the colon, thereby reducing the uptake of ATP. Furthermore, based on our results, it remains possible that intestinal breakdown of ATP to other metabolites such as adenosine did not occur fast enough to allow efficient uptake of these metabolites. Finally, ATP that was released in the small intestine may also have been utilized by the enterocytes lining the intestinal wall, given their poor capacity for de novo purine nucleotide biosynthesis[20]. The fate of ATP after oral administration will be discussed after the results of the second human experiment on oral ATP administration.

The third aim was to compare our results with earlier studies on oral ATP administration. Both our study and the study by Jordan et al.[16] showed no increases in ATP concentration. The fact that our maximum dosage of 5000 mg far exceeded Jordan's et al. dosage of 225 mg would indicate that the dosage of ATP itself has no influence on the uptake of ATP, and that the low dosage of ATP used by these authors was not the cause of the lack of response in systemic ATP concentrations.

The ATP dosages of 90 mg administered in the studies by Bannwarth et al.[17] and Rossignol et al.[18] resulted in a diminished use of rescue analgesics in low back-pain patients. However, the period during which ATP was administered in these studies was much longer than in our acute study: 14 days to 1 month. This makes it difficult to compare our study with the above studies.

The experimental set-up using a comparison between the effectiveness of a direct administration into the upper small intestine via a naso-intestinal tube and an oral administration mode, has been used before in studies by Bours et al.[21, 22]. In these studies in healthy volunteers, the authors investigated the effect of ATP on compromised mucosal barrier function in the small intestine in healthy human

subjects by administering ATP after a short-term nonsteroidal anti-inflammatory drug (NSAID) challenge. Results showed attenuation of the increase in small-intestinal permeability after direct administration through a naso-intestinal tube of 30 mg ATP per kg body weight, i.e. approximately 2 g of ATP in total [21]. This effect of ATP was suggested to be beneficial in reducing small bowel side effects of NSAIDs by preserving mucosal barrier function. In a subsequent study, Bours et al. administered ATP through oral ingestion of two times five enteric-coated (Eudragit® L30D-55) hydroxypropyl methylcellulose capsules, delivering 4 grams of ATP in total [22]. Even though this dose was higher than in the previous study which used a naso-intestinal tube, the same favourable effect was not observed when using the capsules. Possible explanations given by the authors for the lack of this favourable effect included a) the difference in site-specificity of ATP delivery between duodenum and proximal jejunum by the naso-intestinal tube and ileum for the capsules, and b) the difference in timing of ATP release: either preceding (naso-intestinal tube) or following (capsules) the permeability effects induced by NSAIDs.

With regard to the latter, administration methods using rather large single units like 00-sized capsules may be subject to considerable variation in individual intestinal transit times[23]. This was also one of the reasons why we used small enteric coated pellets that have no gastric retention, because their size allows them to pass the pyloric opening together with fluids.

Further conclusions from this experiment were that the used oral ATP pellets are a) easy to ingest, b) likely to target the proximal small intestine, and c) less susceptible to side effects compared to ATP infusion.

Effects of prolonged periods of ATP administration

In order to provide more certainty on whether ATP administration is influenced by the duration of ATP administration, a second oral ATP administration study was set up in which the period of ATP administration was 4 weeks (**Chapter 4**). We used the pellets that resulted in the highest uric acid concentrations in the first study. We investigated how ATP handling in the body is affected by 4 weeks of administration of 250, 1250 or 5000 mg ATP per day. We did this by comparing the effects of a single administration of 5000 mg ATP on days 0 and 28, when in between 0, 250, 1250, or 5000 mg ATP was administered daily. To our surprise, we found no difference in the increase of uric acid between days 0 and 28, suggesting that the handling of ATP is not affected by the repeated administration of ATP.

This is the first study to report on the effects of long-term daily oral administration of high dosages of ATP. Lower dosages of 225 mg have been administered in the study by Jordan et al., but only a marginal effect on exercise was reported[16]. This study also showed no increases in whole blood ATP. In rabbits and rats, ATP that was administered into an isolated portion of the jejunum, resulted in rapid intraluminal transformation into ADP, AMP, adenine and uric acid. When the concentration was measured in the portal vein, the concentrations of ATP, ADP, AMP, adenine and inosine were found to increase. Also the plasma ATP concentration in

the portal blood was found to have increased following introduction of ATP in the jejunum[24, 25].

As mentioned above, Rossignol et al. [17] and Bannwarth et al. [18] reported a diminished use of rescue analgesics in low back-pain patients after oral ATP dosages of 90 mg were administered for a period one month. The fact that we found no change in blood ATP levels after 1, 2, 3 and 4 weeks of ATP administration, even at doses of up to 5 g, shows that study duration cannot explain the paradox between the apparent clinical effect in these studies and our lack of physiological effect on ATP concentrations. Three possible explanations can be given: 1. a possible short term effect of ATP on metabolism which is not picked up by our whole-blood ATP measurement, e.g. due to a strong first-pass effect of the liver, and 2. a different mechanism of the ATP effect, e.g. via local intestinal effects of ATP, or via uric acid or other intermediary metabolites of ATP. As an additional explanation, the possibility of false-positive findings in earlier studies with oral ATP cannot be completely disregarded in view of the mostly subjective outcome measures and the usually modest clinical effects of ATP.

General discussion on oral ATP administration

From the results presented in chapters 3 and 4, we can conclude that ATP in pellets does not reach the systemic circulation in the form of ATP, regardless of the duration of administration. In animal studies, increases in ATP in the portal vein were observed after oral ATP administration, for example by Kichenin et al[25]. We were unable to determine portal vein concentrations in humans because of its invasive nature. Our experiments can therefore give no clue as to where ATP is metabolized (i.e. intestinal lumen, enterocytes, portal circulation, liver, …).

Furthermore, we can conclude from our studies that it is highly unlikely that ATP in a similar oral formulation can achieve better results when lower concentrations than 5000 mg are used. This is in contrast to the positive results that were obtained by 4-week daily administration of Atépadène®, an oral enteric coated supplement containing only 90 mg ATP, to low-back pain patients[17]. Also Rapaport's claim that his formulation prescribing 125 mg ATP per day, which is marketed as PeakATP® in the world of bodybuilding, can induce positive ergogenic effects in sports and exercise, has to be met with skepticism [26]. Several interviews with Rapaport can be found on the website Healthtube [27]. Unfortunately, the claims done in these interviews ascribed to this oral ATP formulation are not backed up by scientific evidence; also our own studies do not provide supportive evidence.

So, one important question still remains: "What is the fate of orally administered ATP?" In order to answer this question, we need to look at all locations in the body through which ATP passes after oral administration. First, in our studies, ATP is administered as enteric coated pellets, which prevents contact with gastric acid and enzymes. Once the coating of the pellets disintegrates due to the increased pH in the small intestine compared to that in the stomach, ATP will be faced with the enzymes present in the intestinal lumen. Currently, no transporters

have been described that could mediate direct translocation of extracellular ATP across biological membranes to be reused in the intracellular compartment for metabolic recycling. Therefore, as was discussed in **chapter 4,** ATP is likely to first be degraded to adenosine in the intestinal lumen. Transporters that mediate membrane translocation of nucleosides, including adenosine, are known to be present at the plasma membrane of most cells, including absorptive epithelia [28]. Two families have been categorized: the concentrative nucleoside transporters (CNT1-3) and the equilibrative nucleoside transporters (ENT1-3)[28-30]. The CNT family facilitates unidirectional transport of nucleosides against their concentration gradient through active cotransport across the membrane coupled to an inwardly directed sodium gradient, whereas ENT mediated transport of nucleosides occurs bidirectionally by facilitated diffusion along their concentration gradient. CNT1 and CNT2, which have been identified in the brush border membrane of the human small intestine, with the highest activity in the jejunal region, could mediate uptake of adenosine into the small intestinal enterocytes. Inside the enterocytes, adenosine could serve as a precursor to intracellular nucleotides, including ATP. Evidence for this has been found in rat intestine in which a > 80% reduction in tissue ATP concentrations was induced by mesenteric ischemia. Direct administration of adenosine into the rat intestine restored ATP concentrations in jejunal tissues to near normal [31].

In our oral ATP administration studies, we observed increases in whole blood uric acid concentrations. Since these increases can be traced directly to the ATP that was administered, it can be speculated that ATP is not solely used by the enterocytes. Studies with i.v. administration of ATP show that adenosine, formed from breakdown of ATP entering the circulation, is highly efficiently taken up by erythrocytes through ENTs and utilsed for ATP resynthesis[32]. This is actually also the regular physiological mechanism by which ATP, after it has been used for extracellular signaling, gets degraded. Membrane-bound and soluble nucleotidases, including those of the E-NTPDase and E-NPP families, ecto-5'-nucleotidase/CD73, and alkaline phosphatases all act to quickly degrade ATP after it is released. In our study, however, no increase in ATP or any other metabolites except uric acid was found. Since the samples we took were from the systemic circulation, the degradation of ATP metabolites to uric acid by xanthine oxidase may either have occurred in the liver or in endothelial cells.

The double-life of uric acid in blood

In our oral ATP administration studies (results are presented in chapters 3 and 4), we found a marked increase in uric acid concentration. As described earlier, partial metabolism in the small intestine and in the liver were likely sources of uric acid, the final product of human purine metabolism. For years, elevated uric acid concentrations have been linked to the development of gout. On the other hand, uric acid is one of the major antioxidants in plasma and may thus also have

a protective role. In recent years this 'uric acid paradox' has renewed interest into uric acid's properties[33-37].

In the first of our two ex vivo studies, we investigated not only uric acid's effect on cytokine production, but also that of the other metabolites of ATP that were identified in Kichenin et al.'s[24, 25] animal studies, such as ADP, AMP, adenosine, inosine, and hypoxanthine. As discussed in **Chapter 5**, several interesting observations were made. First, the slower than anticipated degradation of ATP was due to dilution of the blood. Second, instead of uric acid, hypoxanthine was found to be the most predominant metabolite of ATP when blood was incubated for 24 hours. This effect is likely due to the absence of xanthine oxidase, the enzyme that degrades hypoxanthine further to uric acid. Xanthine oxidase is mainly present on endothelial cells of blood vessels and in the liver[38, 39]. Third, ATP was found to exert an overall anti-inflammatory effect, by attenuating the stimulation-induced release of pro-inflammatory cytokines and increasing the release of anti-inflammatory cytokines. All ATP metabolites had individual effects on the inflammatory reaction that was induced by LPS/PHA.

In the second ex vivo study, we aimed at characterizing the immune response that either uric acid in solution or MSU crystals would have on blood in which an inflammatory response was either already present or not. This study showed that, in unstimulated blood, MSU crystals evoked a far stronger pro-inflammatory response in blood than uric acid crystals. In contrast, when blood was stimulated with LPS/PHA, both MSU crystals and UA crystals exhibited an anti-inflammatory effect, possibly because the applied LPS/PHA concentration had already induced maximum cytokine production. Of note, the unstimulated situation resembles the normal situation in blood. Our results therefore appear to mimick the strong pro-inflammatory reaction induced by MSU crystals mimics the situation in gout patients, in which MSU crystals lie at the heart of the inflammation [40].

Overall conclusions and directions for future research.

Thus, although the results of our two oral ATP administration studies tend to lead to the conclusion that oral ATP administration fails to be as effective as intravenous administration with regard to increasing blood ATP concentrations, some ideas for future research emerge.

A commonly heard argument against supplementation is that the daily administration of 5 gram of ATP stands in sharp contrast to the 60 kilogram of ATP that is being recycled in an average person every day. This argument, however, does not take into account the fact that at any given moment, there is as little ATP in the body as around 50 gram. In that respect, administration of relatively small amounts of ATP (as has been proven by intravenous administration) might immediately improve the rate of de novo synthesis of ATP, instead of its reconstitution from ADP and phosphate.

Another point which our investigation did not fully elucidate, is the fate of ATP in the small intestine, since this is very hard to determine in humans by non-invasive methods. Animal studies, however, showed increased concentrations of ATP, ADP, AMP adenosine, adenine, inosine, and uric acid in plasma from the portal vein after ATP was administered to isolated parts of the small intestine[24, 25]. A promising option would be to administer stable-labeled ATP to individuals with an ileal stoma. By collecting ileal effluents at regular intervals after ATP administration, it would be possible to obtain a valuable insight into the handling of ATP in the small intestine. By identifying the proportion of labeling in the effluent fractions, the source of the specific metabolites could be traced back to the ATP that was originally administered. Drawbacks of this type of investigation would include cost and difficulty of standardization due to altered characteristics of the small intestine after colectomy and between-subject variability in length of the remaining gastro-intestinal tract and the type of infliction.

Our in vitro studies with ATP and uric acid crystals and ATP crystals have shed more light on the inflammatory effects of ATP, its metabolites, MSU crystals and UA crystals. The stimulated whole blood model proved, once again, to be a useful model to study inflammation which closely resembles the in vivo situation. Further studies on the effect of MSU crystals in specialized cell types, like PBMCs, would be valuable. It would also be interesting to study other crystal structures like calcium pyrophosphate dihydrate, in order to find a clue on what exact part of the crystal structure induces inflammation.

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Chapters 8 and 9

Summary

and

Samenvatting

Summary

In this thesis, we investigated the possibilities to orally administer the naturally occurring compound adenosine 5'-trifosfaat (ATP). The goal was to investigate whether oral ATP administration can be less burdensome for patients and whether it can be a simpler and more cost-effective alternative to intravenous ATP administration.

In previous years, intravenous administration studies using ATP have been applied in the palliative treatment of cancer, rheumatoid arthritis and in the treatment of postoperative pain. Depending on the patient group, the goal of this intravenous administration of ATP was to prevent inflammation, pain and fatigue and involuntary weight loss and to improve the quality of life. Especially with respect to logistics, patient burden due to side effects, infusion time and the need for an experienced nurse, the more common use of ATP infusions is fraught with difficulties. Since oral ATP administration lacks these drawbacks, we proposed it to be an alternative mode of administration.

Oral ATP administration distinguishes itself from intravenous administration by the more complex route it has to take to reach circulation. ATP may be degraded to its metabolites in the gastro-intestinal system, portal vein and liver, due to interactions with each of these environments. In order to monitor the reactions of ATP that take place at each step, it is important to be able to measure several metabolites of ATP in blood samples. In chapter 2 an HPLC method is described to simultaneously measure seven metabolites of ATP. The advantages of this method are the stability of the samples, high sensitivity and high throughput speed. We employed this method in both human volunteers studies.

Oral ATP administration studies have been limitedly performed before and those that were carried out, had several drawbacks. First, direct comparison is hampered by the fact that dosages of ATP differed between intravenous and oral studies. An intravenous dosage of 50 $\mu g/kg \cdot min^{-1}$ ATP can be administered safely in a homecare setting, delivering 1680 mg ATP to a 70 kilo person during 8 hours. Earlier oral administration studies, however, only administered dosages of 90 mg per day for a 30 day period. Second, in earlier oral administration studies, results were based on the outcomes of questionnaires or physical exercises.

Both human administration studies carried out in this experiment were done in healthy volunteers and had the goal to mimic earlier intravenous ATP administration studies, regarding dosage and sampling. In **chapter 3,** ATP is administered either as microcapsules (pellets) or through a duodenal tube. The pellets are enteric coated 1 mm spheres that are designed to controlled release ATP in two distinct areas of the small intestine, i.e. either proximal (the duodenum) or distal (the ileum). Results of measurements in blood samples collected after both modes of administration indicated that only plasma uric acid concentrations, but not concentrations of ATP or metabolites, increased. In case of the proximal-release pel-

lets, ATP was likely to have been released near the duodenum or jejunum. The distal-release pellets showed less pronounced increases of uric acid concentrations and were thus deemed not a favorite choice for further investigation. In addition, in this singe administration study, no side-effects were reported and the pellets were easy to ingest.

In **chapter 4** we present the results of a study in healthy volunteers using the proximal-release pellets. The goal of this study was investigate the hypothesis that a single administration of ATP in the form of pellets was just insufficient to increase ATP concentrations in blood. We administered either 0, 250, 1250 or 5000 mg of ATP per day for a period of 28 days. On days 0 and 28 of the study, all participants received 5000 mg ATP (a replication of the study described in chapter 3) and the goal was to see whether the period in between the administrations has an effect on the way these high ATP dosages are handled by the body.

Similar to the first single administration study, no changes in ATP or metabolite concentrations were observed, except for uric acid. Between days 0 and 28, no distinction could be made regarding the way the single 5000 mg administration of ATP on these days affected the blood ATP and uric acid concentration. We also found no effects on the plasma ATP concentration. Although administration of ATP through microcapsules was well tolerated, easily ingested and the contents were delivered to the small intestine, it does not have the same effect as intravenous administration. We conclude that oral administration by means of microcapsules in healthy volunteers can not be used as an alternative to intravenous administration. Furthermore, we doubt the correctness of the results presented in earlier studies form other groups claiming to have observed increased ATP concentrations after oral administration of far lower ATP dosages.

In **chapter 5** we investigated the effects of ATP and metabolites on inflammatory responses in blood ex vivo. In freshly drawn blood, an inflammatory response was induced by adding LPS and PHA, two compounds that initiate inflammation. Simultaneously, ATP was added, and during 24 hours, the degradation profile of ATP was recorded by taking samples at several timepoints. The results showed a constant concentration of the final degradation product in humans of ATP: uric acid. Some intermediate metabolites, like AMP and hypoxanthine, in the mean time, increased strongly. This indicates that degradation of ATP outside of the body, stops prematurely, possibly because the appropriate enzymes are not present. ATP (and metabolites) had an in general anti-inflammatory effect, as was proven by the decreased concentrations of the cytokines TNF-α, IFN-γ and IL-1β, and the increased concentration of IL-10.

In the study described in **chapter 6**, wholeblood was again incubated with LPS and PHA to induce an inflammatory response. This time, however, instead of ATP, various concentrations of uric acid and monosodium urate (MSU) crystals were used to incubate the blood with. We were led to this investigation by the increased uric acid concentrations that we observed in both oral administration studies. Uric acid is mainly known as the causative agent of gout, but the last years,

its antioxidant properties gained attention. The goal was to investigate the way uric acid influences inflammatory markers and antioxidant capacity in whole blood.

Since the monosodium salt of uric acid (MSU) is present in the joints of gout patients as sharp needles and is being regarded as the direct cause of the often painful inflammation in these joints, we specifically opted to use these in our study. The results showed that the various concentrations of MSU crystals had a strong pro-inflammatory response in blood, whereas 'normal' uric acid did not. When simultaneously incubated with LPS and PHA to evoke an inflammatory response, both MSU and normal uric acid had an anti-inflammatory effect. This may be due to the already maximized stimulation in the blood caused by LPS and PHA impeding an even further increase.

The general conclusions of this thesis are:

i) Oral administration of ATP by means of enteric coated pellets does not cause an increase in circulatory ATP concentrations. This finding has been done first in a single administration study and has been replicated in a repeated 28-day daily administration study with dosages of up to 5000 mg per day. The observed increase in uric acid is likely caused by degradation of ATP to uric acid at some point after administration.
ii) The pellets we used were easily ingested and no side effects were observed. The effectiveness of the pellets is comparable to the administration of ATP dissolved in water by means of a duodenal tube directly into the duodenum.
iii) We were, however, unable to replicate the increased ATP concentrations that were observed in some studies using far lower ATP concentrations. Given the thorough design of our experiments, we seriously doubt the claims these studies have made.
iv) ATP has anti-inflammatory properties in LPS-PHA stimulated blood ex vivo.
v) Monosodium urate (MSU) crystals, the causative agent of gouty arthritis, causes a stronger pro-inflammatroy response than normal uric acid.

Samenvatting

In dit proefschrift is de mogelijkheid onderzocht, de lichaamseigen stof adenosine 5'-trifosfaat (ATP) oraal toe te dienen. De achterliggende gedachte hiervan was dat orale ATP toediening een patiëntvriendelijker, eenvoudiger en goedkoper alternatief zou kunnen bieden voor intraveneuze toediening van ATP.

Intraveneuze toediening van ATP is in de afgelopen jaren onder meer toegepast in de palliatieve fase van kanker, bij reumatoïde artritis en bij postoperatieve pijn met als doel (afhankelijk van de patiëntengroep) het remmen van inflammatie, pijn en vermoeidheid, het tegengaan van gewichtsverlies en het verbeteren van de levenskwaliteit. Op de gebieden van logistiek, belasting voor de patiënt door bijwerkingen en duur van de infusen en de benodigde hulp van ervaren verpleegkundigen, kleven er echter beperkingen aan intraveneuze toediening, welke de toepassing van behandelmethoden met ATP op grotere schaal in de weg staan. Orale toediening van ATP heeft deze nadelen niet, en werd daarom aan het begin van het onderzoek voorgesteld als alternatieve toedieningwijze.

De route die ATP na orale toediening moet afleggen om uiteindelijk in de bloedcirculatie te komen, is complexer dan de route na intraveneuze toediening. Bij iedere stap in deze route, het maag-darmkanaal, de poortader en de lever, zal het toegediende ATP een interactie met het lichaam aangaan, steeds met de bijbehorende mogelijkheid van chemische verandering, zoals de vorming van metabolieten. Om een zo goed mogelijke inschatting te kunnen maken van wat er gebeurt met ATP na orale toediening, is het belangrijk om verscheidene metabolieten van ATP in bloedmonsters te kunnen meten. Daarvoor beschrijven we in **Hoofdstuk 2** een vloeistofchromatografische methode om in bloedmonsters in één meting zowel ATP als zeven metabolieten van ATP te kunnen meten. De eigenschappen van deze methode omvatten: stabiliteit van de bij proefpersonen afgenomen samples, een hoge gevoeligheid en een hoge snelheid van de metingen. Deze methode is bij de proefpersoonstudies in dit proefschrift gebruikt om de spiegels van ATP en metabolieten in bloedsamples te meten.

Tot op heden hebben nog maar weinig studies de effectiviteit van orale ATP toediening bij mensen onderzocht. De studies die uitgevoerd zijn, hadden op verschillende gebieden beperkingen. Ten eerste bemoeilijkt het verschil in dosering tussen intraveneuze en orale toediening een onderlinge vergelijking. De doseringen die bij een regulier infuus in de thuissituatie veilig kunnen worden gebruikt liggen op 50 $\mu g/kg \cdot min^{-1}$. Een persoon van 70 kilogram krijgt bij een 8-uurs infuus dan 1680 mg ATP toegediend. Bij eerdere orale toedieningstudies echter, werd gedurende een periode van 30 dagen, slechts een dosering van 90 mg per dag gegeven. Ten tweede zijn er bij eerder uitgevoerde orale toedieningstudies geen bloedmonsters verzameld, maar zijn als uitkomstmaten uitsluitend vragenlijsten of fysieke inspanningen toegepast.

De in dit proefschrift beschreven humane studies vonden plaats bij gezonde proefpersonen en hadden tot doel, qua dosering en qua sampling zo nauw mogelijk aan te sluiten bij eerdere intraveneuze toedieningsstudies van ATP. In **hoofdstuk 3** werd de orale opname van ATP onderzocht bij de toediening via microcapsules of een duodenaalsonde (een dun slangetje via de neus rechtstreeks naar het duodenum). De microcapsules bestaan uit bolletjes met een diameter van ongeveer 1 mm die zijn omhuld met een laagje, dat afhankelijk van de pH waaraan het wordt blootgesteld de inhoud beschermt tegen de invloeden van het spijsverteringsstelsel. Bij lage pH waarden, zoals deze in de maag voorkomen, blijft de omhulling intact. Na de maag stijgt de pH-waarde naar ongeveer pH 6.0 in het duodenum tot ongeveer pH 7.5 in het ileum. In deze studie hebben we ervoor gekozen om ATP te verpakken in twee soorten microcapsules, en wel capsules die hetzij proximaal (duodenum) hetzij distaal (ileum) in de dunne darm de inhoud laten vrijkomen. De effecten van deze twee soorten microcapsules hebben we vergeleken met de toediening van ATP opgelost in water via een duodenaalsonde. Metingen in afgenomen bloed lieten bij beide vormen van toediening uitsluitend stijging zien van het urinezuurgehalte, maar niet van ATP of andere metabolieten van ATP. Tevens werden er geen bijwerkingen bij deze eenmalige toediening gerapporteerd en waren de microcapsules makkelijk in te nemen.

Hoofdstuk 4 presenteert de resultaten van een studie bij gezonde vrijwilligers waarbij gebruik werd gemaakt van de microcapsules met het proximale vrijgifteprofiel. Het doel van deze studie was te kijken naar het effect van een 28-daagse dagelijkse toediening van ATP in 4 verschillende doseringen op de opname van een eenmalige dosering ATP. Hiertoe kregen alle deelnemers op dag 0 en dag 28 een hoge dosering (5000 mg) ATP en in de tussenliggende dagen ofwel 0, 250, 1250 of 5000 mg ATP per dag. Hiermee zijn dagen 0 en 28 te zien als een herhaling van de eenmalige toediening zoals die in hoofdstuk 3 werd vermeld en ligt het verschil in de tussenliggende periode.

De resultaten van deze studie lieten wederom geen veranderingen in de bloedspiegels zien van ATP of metabolieten buiten urinezuur. Tevens zorgde de periode van 4 weken dagelijkse toediening van ATP er niet voor dat er een verschil tussen het effect van de hoge ATP dosis op dagen 0 en 28 ontstond. In deze studie werden ook geen effecten op de concentratie ATP in plasma. Uit deze studie kan worden geconcludeerd dat, hoewel de microcapsules a) hun inhoud in de dunne darm vrij lieten komen, b) goed werden getolereerd en c) gemakkelijk in te nemen waren, deze microcapsules niet hetzelfde effect hadden als de intraveneuze toediening en dus in deze vorm ook niet als alternatief hiervoor kunnen dienen. Eerder door buitenlandse groepen gerapporteerde stijgingen van ATP in de circulatie na orale ATP-toediening moeten dus in twijfel worden getrokken.

In **hoofdstuk 5** beschrijven we een studie waarin de effecten van ATP en afbraak producten van ATP op een ontstekingsreactie in bloed ex vivo (dwz in een reageerbuis) bestudeerd werden. Daartoe werd in vers afgenomen bloed een ontstekingsreactie geïnduceerd door hieraan twee ontstekings-stimulerende stoffen

(LPS en PHA) toe te voegen. Tegelijkertijd werd in dit volbloedmodel ATP toegevoegd, en werd het afbraakprofiel van ATP tijdens de incubatie gevolgd. De resultaten lieten onder meer zien dat gedurende de 24-uurs incubatie van het bloed met ATP, de concentratie van urinezuur als eindproduct van de afbraakcascade constant bleef, terwijl enkele tussenliggende afbraakproducten van ATP zoals AMP en hypoxanthine juist wel sterk in concentratie stegen. Dit resultaat duidt erop dat ATP buiten het lichaam niet tot urinezuur wordt afgebroken. ATP (en afbraakproducten) hadden in het algemeen een ontstekingsremmend effect, zoals bleek uit remming van de cytokines TNF-α, IFN-γ en IL-1β en stimulering van IL-10.

In de studie beschreven in **hoofdstuk 6** is bloed, dat eveneens eerst met LPS en PHA gestimuleerd werd, geïncubeerd met verschillende concentraties urinezuur en urinezuurkristallen. De achtergrond van deze studie was de bevinding in beide orale toedieningstudies van ATP, dat een significante stijging van urinezuur optrad. Urinezuur staat van oudsher bekend als de veroorzaker van jicht, maar de laatste jaren zijn aan urinezuur tevens antioxidant eigenschappen toegeschreven. Gezien de stijging van urinezuur in de orale toedieningsexperimenten, wilden we de invloed van urinezuur op inflammatie en antioxidant-capaciteit nader onderzoeken.

Daarbij werd gekozen voor kristallen van het zout van urinezuur (monosodiumuraat of MSU) omdat dit MSU als scherpe naaldjes in de gewrichten van jichtpatiënten voorkomt en beschouwd wordt als de directe oorzaak van de vaak pijnlijke ontstekingen in de gewrichten bij deze patiënten. Uit de resultaten bleek, dat de verschillende concentraties MSU inderdaad een sterke pro-inflammatoire reactie in het bloed veroorzaakten, terwijl 'normaal' urinezuur niet dit effect had. Wanneer in het bloed echter een ontstekingsreactie werd opgeroepen met LPS en PHA, dan zorgden zowel MSU als normaal urinezuur voor een remming van de ontsteking, mogelijk als gevolg van het feit dat LPS en PHA reeds de maximale stimulatie in het bloed hadden veroorzaakt waardoor dus geen verdere stijging kon optreden.

Algemene conclusies van dit proefschrift zijn:

i) Orale toediening van ATP in de vorm van microcapsules met een pH-gevoelige omhulling aan gezonde personen leidt niet tot een stijging van ATP in het bloed in de circulatie. Deze bevinding geldt zowel voor eenmalige als herhaalde toediening gedurende 28 dagen van ATP in doseringen tot 5000 mg per dag. Wel werd een stijging van de concentratie urinezuur gevonden, wat wijst op afbraak van ATP tot urinezuur in het lichaam.
ii) De gebruikte microcapsules zijn makkelijk in te nemen en we constateerden geen bijwerkingen. De effectiviteit van de microcapsules is te vergelijken met de effectiviteit van toediening van ATP middels een oplossing van ATP in water rechtstreeks in het duodenum via een duodenaalsonde.
iii) De positieve gerapporteerde effecten van orale toediening van ATP in incidentele studies met veel lagere ATP-doseringen hebben wij niet kunnen repliceren.

Gezien de grondige uitvoering van ons onderzoek met uitgebreide dosisrespons tests, trekken we de claims van deze eerdere studies in twijfel.

iv) ATP werkt ontstekingsremmend in LPS-PHA gestimuleerd bloed ex vivo.

v) Kristallen van monosodiumuraat (MSU), dat in gewrichten van jichtpatiënten voorkomt, zorgen in volbloed dat gedurende 24 uur werd geïncubeerd voor een sterkere pro-inflammatoire reactie dan normaal urinezuur.

Dankwoord

In dit stukje wil ik graag iedereen bedanken die me heeft geholpen, geïnspireerd en gesteund om hier vandaag dit boekje te kunnen verdedigen. Uiteindelijk heb ik het boekje toch nog af kunnen ronden, hoewel dat tijdens de laatste periode in combinatie met mijn eerstegraads lerarenopleiding erg veel inspanning heeft gekost.

Ten eerst mijn promotor prof. dr. Bast. Aalt, bedankt voor al je hulp bij het meedenken over de proefopzet, het formuleren van sommige passages in de artikelen en het snel nalezen van de opnieuw te submitten stukjes. Gezellig waren ook onze wekelijkse overleggen met Pieter en Ilja. Door je gastvrijheid had ik het gevoel dat ik altijd binnen kon stappen in je kamer voor advies of een praatje. Bedankt voor je vertrouwen in mij en in het toch nog afronden van mijn proefschrift.

Ten tweede mijn copromotoren Pieter en Ilja. Pieter, jouw uitstekende onderzoeksmentaliteit heeft mij zeker geholpen om door te zetten. De snelheid waarmee jij keer op keer verbeteringen en inkortingen in mijn stukken hebt doorgevoerd is echt geweldig! Jij bent de spin in het web van het ATP-onderzoek binnen de afdeling en bleef actief bezig met het zoeken van nieuwe mogelijke toepassingen.

Ilja, met jou was het altijd prima samenwerken. We hebben hele periodes met zijn tweeën overlegd en dat liep altijd gesmeerd. Je was onmisbaar bij het uitwerken van de proefopzet en de meetmethode. Met de HPLC hebben we samen een werkende methode opgezet door veel verschillende kolommen uit te proberen. Ook heb je me regelmatig aangemoedigd om nog even door te zetten.

Mijn tweede promotor, prof. dr. Cohen Stuart (Martien) wil ik graag bedanken voor zijn deskundige inbreng op onze regelmatige overlegmomenten. Dit heeft me zeker geholpen om nog eens vanuit een ander perspectief naar de resultaten te kijken.

Mijn aio-periode was zeker een periode in mijn leven waar ik met veel plezier op terugkijk. Na voor het eerst op mezelf te zijn gaan wonen in een klein gehorig kamertje in het Belgische Vroenhoven, was ik blij om na twee jaar eindelijk tussen de uni en het centrum van Maastricht te kunnen gaan wonen. Maastricht is echt een van mijn lievelingssteden geworden. Ik heb heel erg genoten van alle gezellige momenten die we op de afdeling en zeker ook daarbuiten beleefd hebben.

Ruben, je bent echt een van de leukste personen die ik ooit heb ontmoet. Het was geweldig om met jou zo'n drie keer per week naar de badminton te gaan, bijna 3 jaar lang. Daarnaast was het super om ons te verbazen over echt Amerikaanse dingen (kniehoge witte sokken in sandalen) tijdens onze vakantie bij Sue in Daytona Beach, Florida, samen met Hilde en Bea. Daarna een onvergetelijke reis door China en Tibet, waar ik eindelijk enkele van jouw favoriete gerechten uit de Chinese keuken eens in het echt kon uitproberen. Daarnaast denk ik graag terug aan

de vele gezellige koffie's, etentjes en feestjes die we samen hebben bezocht en/of georganiseerd. Je woont en werkt nu in Amerika samen met Hilde. Daarbij wil ik je veel succes wensen, maar dat zal wel lukken, want jij weet overal je thuis te maken.

Hilde, kleine maar fijne Belgische meid, jij zat ook al vanaf het begin in het groepje. Het was altijd erg gezellig om samen bij jou te eten. Hopelijk hebben we je wat leuke Nederlandse dingen bijgebracht. Nu in Amerika, maar misschien ooit toch nog terug in België om daar carrière te maken? Bea, bedankt voor de gezellige avonden en voor de leuke wandelingen in Maastrichts' buitengebied. Samen met Hilde en Ruben was de vakantie in Daytona Beach geweldig. Ik hoop jullie Amerika-gangers gauw weer eens te zien, misschien wel in the US of A.

Hola Nuria! Ik was blij om jou als nieuwe vaste kamergenoot te krijgen nadat je al eens een tijdje een korte periode er was. Je positieve energie en tomeloze inzet zijn ongeëvenaard. Ook buiten de uni denk ik graag terug aan het stappen, carnavallen enz. Hopelijk vind je snel een mooie voortzetting van je werk in Maastricht. Adiós! Fijn dat je nu mijn paranimf wilt zijn!

Esther, trotse Sjeng, ik was blij dat je na je stage voor vast het TOX-lab kwam ondersteunen. Thanx voor de superleuke en leerzame periode, de gezellige stapavonden, borrels en uitvoeringen met de MVB.

Mirjam, het gezelligste contactpunt van Epi, je lach werkt aanstekelijk, bedankt voor de leuke koffiekwartiertjes, hulp bij statistiek en leuke tripjes. Martijn, je stond altijd klaar met tips over ATP. Anke, bedankt voor de sfeervolle tijd in Denemarken na het ATP-congres.

Raffaele, crazy Italiano, zo'n sociaal iemand zie je zelden, Thanx voor je humor en gezelligheid. Natuurlijk wil ik ook iedereen van Tox bedanken: Saskia en Liesbeth (mijn eerste kamergenoten), Roger (gezellige vent, bedankt voor je hulp), Els (bedankt voor je hulp bij alle technieken), Guido, Agnes, Antje, Hilde, Marc, Marie-José, Jiska, Gert-Jan, Erik en Max.

Ook wil ik alle andere collega's van Farmacologie en Toxicologie en van Epidemiologie bedanken voor de leuke tijd.

Het op één na laatste woord wil ik richten aan mijn allerliefste moeder. Bedankt voor alle steun die je me altijd hebt gegeven. Met zijn tweetjes hebben we het altijd goed gehad en ik ben blij dat je nu nog dichtbij woont.

Het laatste woord is voor mijn liefie. Er was een donder, een bliksem, een slag toen ik je zag. Lieve Kathelijne, met jou aan mijn zij is mijn leven compleet. Jij maakt me echt zooo gelukkig. Ik kijk nu al uit naar onze grote dag in september. Onze toekomst wordt geweldig samen!

Erik

About the author

Erik Coolen was born on April 26 1981 in Tilburg, The Netherlands. After obtaining his secondary school diploma (VWO) in 1999 at the Cobbenhagen College in Tilburg, he studied Biology at the Radboud University in Nijmegen, in 2004. His major's were in medical biology – with internships at the department of Psychoneuropharmacology of the Radboud Medical Faculty and at the department of Molecular Pharmacology at Schering-Plough in Oss. After obtaining his Master's degree, Erik worked as a PhD student at the Department of Epidemiology and the Department of Pharmacology and Toxicology of Maastricht University. The results of the project on oral ATP administration are presented in the present thesis. Since 2010, Erik is studying at Radboud University Nijmegen to achieve a permit to work as a secondary school biology teacher.

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