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ATP inhibits hydroxyl radical formation and the inflammatory response of stimulated whole blood even under circumstances of severe oxidative stress

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Abstract

We recently reported that Adenosine-5′-triphosphate (ATP) is able to inhibit the inflammatory reaction in stimulated whole blood. Many diseases, in which inflammatory reactions are involved, are associated with oxidative stress. In the present study, we therefore, investigated the effect of ATP on cytokine release in stimulated whole blood under conditions of oxidative stress, as simulated by pre-incubation of blood with hydrogen peroxide (H_2O_2). In the presence of H_2O_2, ATP at concentrations of 100 and 300 μM inhibited Tumour Necrosis factor-alpha (TNF-α) release and stimulated IL-10 release in LPS-PHA stimulated whole blood. Moreover, electron spin resonance (ESR) measurements showed that ATP and its breakdown product Adenosine-5′-diphosphate (ADP) attenuated spin trap-hydroxyl radical adduct formation in the Fenton reaction. Our results demonstrate that even in circumstances of severe oxidative stress, ATP has marked anti-inflammatory properties in stimulated whole blood. Moreover, the inhibition of the hydroxyl radical ESR signal indicates a direct attenuation of oxidative stress by ATP.

Keywords: *ATP, cytokines, blood, LPS, oxidative stress, hydroxyl radicals*

Abbreviations: *ADP, Adenosine-5′-diphosphate; AMP, Adenosine-5′-monophosphate; ATP, Adenosine-5′-triphosphate; COPD, Chronic obstructive pulmonary disease; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; ESR, Electron spin resonance spectroscopy; H_2O_2, Hydrogen peroxide; IL-10, Interleukin-10; IL-6, Interleukin-6; LPS, Lipopolysaccharide; NFκB, Nuclear factor-kappaB; ·OH, Hydroxyl radical; PHA, Phytohemagglutinin; ROS, Reactive oxygen species; SEM, Standard error mean; TLR-4, Toll-like receptor 4; TNF-α, Tumor necrosis factor-alpha*

Introduction

There is evidence that oxidative stress, defined as an unbalance between increased exposure to oxidants and decreased antioxidant capacities, plays an important role in the pathogenesis and progression of chronic diseases such as chronic obstructive pulmonary disease (COPD) [1–4], cancer [5,6], neurological diseases [7,8] such as Parkinson, Alzheimer and amyotrophic lateral sclerosis (ALS) and in cardiovascular diseases [9]. Also radiotherapy, a commonly used therapy for cancer, is well known to cause DNA damage through the production of reactive oxygen species (ROS); this intense formation of ROS is thought to cause a relative overshoot in ROS, which overwhelms the enzymatic and non-enzymatic antioxidant protection [10]. Oxidative stress, and so an increased level of ROS, has been

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considered as a central event in the mediation of inflammatory responses through the activation of transcriptions factors such as nuclear factor-kappaB (NFκB) and activator protein-1 (AP-1), which trigger the expression of pro-inflammatory mediators [11,12].

Extracellular adenosine-5′-triphosphate (ATP) is an important modulator of immune cell function [13–17]. ATP can be released from the cytoplasm of several cell types and interacts with specific purinergic (P1 and P2) receptors, which are present on the surface of many cells. In contrast with the general notion that ATP has predominantly pro-inflammatory effects in isolated cell systems *in vitro* [13,14,18], we recently demonstrated that ATP inhibits the inflammatory response in stimulated whole blood, as indicated by inhibition of the release of the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) and stimulation of the anti-inflammatory cytokine, interleukin-10 (IL-10) [19]. During incubation with hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$) are formed. Since $^{\cdot}OH$ stimulate the inflammatory response through activation of transcription factors, this could influence the previously observed anti-inflammatory properties of ATP. At the same time, oxidative stress can affect the binding of ligands to membrane receptors, such as betaadrenergic receptors and muscarinic receptors, as well as the coupling of receptors to G-proteins or the effect of second messengers [20]. Based on these findings, the receptor mediated anti-inflammatory properties of ATP might well be altered under conditions of induced oxidative stress.

The aim of the present study was to determine the effect of ATP on cytokine release in whole blood under circumstances of oxidative stress. For this purpose, we utilized a model of whole blood stimulated with lipopolysaccharide (LPS) and phytohemagglutinin (PHA), as published earlier [19], in combination with H_2O_2 pre-incubation in order to induce oxidative stress. Appropriate control experiments suggested a scavenging effect of ATP on $^{\cdot}OH$, which was further investigated by electron spin resonance (ESR) measurements.

Materials and methods

Chemicals

Purified PHA HA16 was from Murex, Dartford, UK. Human TNF-α (7300 pg/ml), human IL-10 (4000 pg/ml) and human IL-6 (4500 pg/ml) were obtained from CLB/Sanquin, The Netherlands. RPMI 1640 medium containing L-glutamine was obtained from Gibco, UK. ATP disodium salt was purchased from Calbiochem, USA. Thirty percent H_2O_2 was obtained from Merck. All other chemicals were from Sigma Chemical Company (St Louis).

Incubation conditions

Blood was collected from eight healthy volunteers (age range: 25–45 years; five women and three men) in heparin containing vacutainer tubes (Vacutainer, Becton-Dickinson, 170 I.U). In all the experiments, blood was stored on ice and the incubations were started within 1 h after blood collection [19]. Whole blood was aliquoted into 24-well sterile plates and diluted 1:4 with RPMI 1640 (supplemented with L-glutamine). To induce cytokine production, PHA and bacterial LPS were added to whole blood at 1 and 10 μg/ml, respectively. After addition of H_2O_2, ATP and the stimulants LPS and PHA, the plates were incubated in 5% CO_2 at 37°C for 24 h. Cell-free supernatant fluids were then collected by centrifugation (6000 rpm, 10 min at 4°C) and stored at −20°C until tested for presence of cytokines by ELISA.

All incubations were performed in triplicate. H_2O_2 and ATP were dissolved in RPMI 1640 culture medium. H_2O_2 was added to the blood in a final concentration of 1, 5 and 10 mM in the first 15 min incubation step. After this pre-incubation, ATP was added to the blood in a final concentration range of 1–300 μM in the 30 min incubation step. Blood was pre-incubated with H_2O_2 at 5% CO_2 and 37°C for 15 min before incubation with ATP. The blood was incubated for 30 min with ATP prior to stimulation with LPS-PHA for 24 h also at 5% CO_2 and 37°C.

ELISA measurement

All cytokines were quantified by means of 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. Monoclonal antibodies specific for each component were pre-coated overnight at room temperature in 96-well polystyrene microtiter plates. Standards and samples were added into the wells and then incubated for 1 h at room temperature. The antibody on the microtiter plate then captured the cytokine present in a measured volume of sample or standard, and non-bound material was removed by washing. Subsequently, a biotinylated second monoclonal antibody for each of the components was added and incubated for 1 h at room temperature. Following a washing to remove unbound antibody-enzyme reagents, horseradish peroxidase (HRP)-conjugated streptavidin, which binds onto the biotinylated side of the cytokine complex, was added to the wells and incubated for 30 min at room temperature. After removal of the non-bound HRP conjugate by washing, a substrate solution was added to the wells and incubated for 30 min at room temperature. Color development was stopped by addition of sulfuric acid and the intensity

of the color was measured by a microtiter plate reader (absorbance at 450 nm). The absorbance was transformed to cytokine concentrations (pg/ml) using the standard curve. The sensitivity for TNF-α, IL-10 and IL-6 was, respectively, 4–6, 3–5 and 0.5–1 pg/ml.

Electron spin resonance spectroscopy

ESR studies were performed at room temperature using a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity cavity and 12 kW power supply. The following instrument conditions were used: scan range, 60 G; center magnetic field, 3490 G; modulation amplitude, 1.0 G; microwave frequency, 9.86 GHz; time constant, 40.96 ms, scan time, 20.48 ms and number of scans, 50. OH radicals were generated by the Fenton reaction, and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was used for trapping ˙OH. Fifty microliters of 10 mM H_2O_2, 50 μl of 250 mM DMPO, 50 μl milliQ (control) or sample and 50 μl of 5 mM $FeSO_4$/5 mM EDTA were mixed and transferred to a capillary glass tube. DMPO-OH spin adducts were measured after 2 min by ESR. Quantification of the spectra (in arbitrary units) was performed by peak integration using the WIN-EPR spectrum manipulation program.

Statistical analyses

The effect of H_2O_2 on the cytokine release in the absence/presence of ATP was compared to the control (no H_2O_2/no ATP) using Wilcoxon's signed rank test. Differences between ESR signal peak areas were analyzed by Student *t*-test. The two-tailed *P* values of 0.05 or less were considered statistically significant. Results are reported as means ± SEM.

Results

Figure 1 shows the effect of ATP on the TNF-α release in LPS-PHA stimulated whole blood in the absence and presence of different concentrations of H_2O_2. ATP in the absence of H_2O_2 concentration dependently and significantly inhibited TNF-α release from LPS-PHA stimulated whole blood at 100 and 300 μM by 32 ± 9% (mean ± SEM) and 65 ± 5%, respectively. Lower concentrations of ATP (1, 3, 10 and 30 μM), did not affect TNF-α release (data not shown). As seen in Figure 1, ATP in the presence of 1 mM H_2O_2 also significantly inhibited TNF-α release from LPS-PHA stimulated whole blood by 24 ± 9% at an ATP concentration of 100 μM and by 52 ± 6% at a concentration of 300 μM ATP. In the presence of 5 and 10 mM H_2O_2, the inhibition of TNF-α release by 100 μM of ATP was no longer significant, but 300 μM of ATP still significantly inhibited TNF-α release by 46 ± 5 and 53 ± 8%, respectively. ATP concentrations below 100 μM, when combined with the three different concentrations of H_2O_2, did not affect TNF-α release (data not shown).

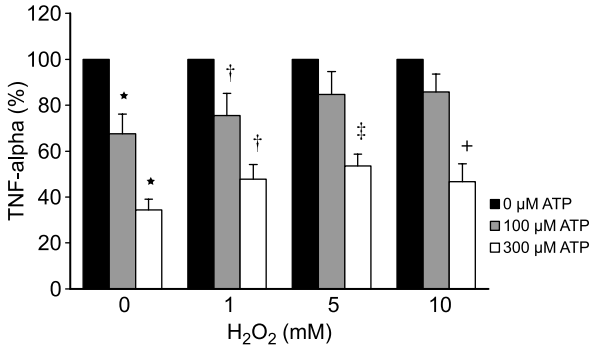

Figure 1. Effect of ATP on LPS-PHA-induced TNF-α secretion in whole blood from healthy subjects in the presence of different concentrations of H_2O_2. Whole blood was preincubated for 15 min with H_2O_2, followed by incubation with ATP for 30 min and stimulation with LPS and PHA for 24 h. TNF-α released into the supernatants was analyzed using the ELISA method. Results are expressed as percentage, 100% being the TNF-α release under stimulation by LPS-PHA without ATP in the presence of the same concentration (0, 1, 5 and 10 mM) H_2O_2 (= control). Data are mean ± SEM in eight subjects; each experiment was performed in triplicate. $^{\star}P < 0.05$ when compared to control (100% = no ATP and no H_2O_2). $^{\dagger}P < 0.05$ when compared to control (100% = no ATP and 1 mM H_2O_2). $^{\ddagger}P < 0.05$ when compared to control (100% = no ATP and 5 mM H_2O_2). $^{+}P < 0.05$ when compared to control (100% no ATP and 10 mM H_2O_2).

ATP, in the absence of H_2O_2 significantly increased IL-10 release from stimulated whole blood at 100 and 300 μM by 93 ± 56 and 166 ± 71%, respectively (Figure 2). Also after pre-incubation with 1 and 5 mM H_2O_2, ATP significantly increased IL-10 release: at 100 μM ATP, the increase in IL-10 relative to the control condition (H_2O_2 but no ATP) amounted to 34 ± 15 and 80 ± 11% at 1 and 5 mM H_2O_2, respectively, and at 300 μM ATP to 114 ± 29 and 79 ± 24%, respectively. Again, at 10 mM H_2O_2, only the highest concentration of ATP had a significant stimulatory effect on IL-10 release of 315 ± 102%. After 5 mM H_2O_2 pre-incubation, no difference between the effect of 100 and 300 μM ATP was observed. ATP concentrations below 100 μM had no effect on IL-10 release, regardless of whether H_2O_2 was absent or present (data not shown). ATP had no effect on IL-6 release from LPS-PHA stimulated whole blood either in the absence or presence of 1, 5 and 10 mM H_2O_2 (data not shown).

H_2O_2, added to whole blood in the absence of both ATP and LPS-PHA, seemed to induce a dose-dependent stimulation of TNF-α, IL-10 and IL-6 release, which did, however, not reach statistical significance when compared with the control condition (no H_2O_2) (data not shown). In the presence of LPS and PHA, 10 mM H_2O_2 showed a significant inhibition on LPS-PHA-induced TNF-α release (decrease from 6487 ± 2469 to 4091 ± 2206 pg/ml,

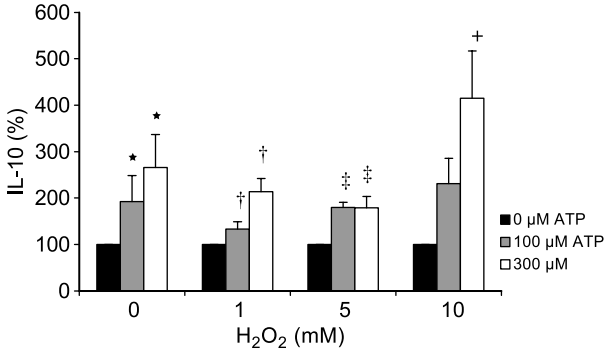

Figure 2. The effect of ATP on LPS-PHA-induced IL-10 secretion in whole blood from healthy subjects in the presence of different concentrations of H_2O_2. Whole blood was preincubated for 15 min with H_2O_2, followed by incubation with ATP for 30 min and stimulation with LPS and PHA for 24 h. IL-10 released into the supernatants was analyzed using the ELISA method. Results are expressed as percentage, 100% being the IL-10 release under stimulation by LPS-PHA without ATP in the presence of the same concentration (0, 1, 5 and 10 mM) H_2O_2 (= control). Data are mean ± SEM in eight subjects; each experiment was performed in triplicate. $^{\star}P < 0.05$ when compared to control (100% = no ATP and no H_2O_2). $^{\dagger}P < 0.05$ when compared to control (100% = no ATP and 1 mM H_2O_2). $^{\ddagger}P < 0.05$ when compared to control (100% = no ATP and 5 mM H_2O_2). $^{+}P < 0.05$ when compared to control (100% no ATP and 10 mM H_2O_2).

$P < 0.05$), while IL-10 and IL-6 release were not influenced (data not shown).

To investigate the direct effects of ATP on ·OH formation, we tested the scavenging effects of ATP on ·OH by means of ESR measurements. As shown in Figure 3, even ATP concentrations as low as 100 μM showed an inhibiting effect on DMPO-OH spin adduct formation generated by the Fenton reaction. Moreover, a concentration dependent inhibition of DMPO-OH spin adduct formation was observed by

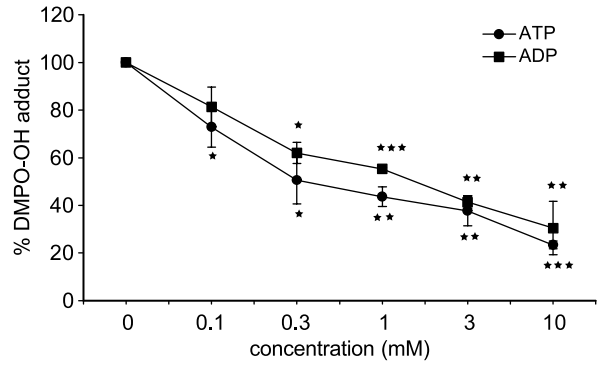

Figure 3. Inhibition by ATP and ADP of DMPO-OH spin adduct formation. After addition of ATP or ADP (0.1–10 mM), DMPO-OH spin adducts were analyzed using ESR spectrometry. ·OH were generated by the Fenton reaction (10 mM H_2O_2 in combination with 5 mM $FeSO_4$-EDTA), and DMPO was used for trapping ·OH. Values were means of triplicate determinations, and 100% is the percentage of DMPO-OH spin adducts formed when no ATP or ADP is present (control). Statistically significant deviations relative to control are represented by $^{\star}P < 0.05$, $^{\star\star}P < 0.01$ and $^{\star\star\star}P < 0.001$.

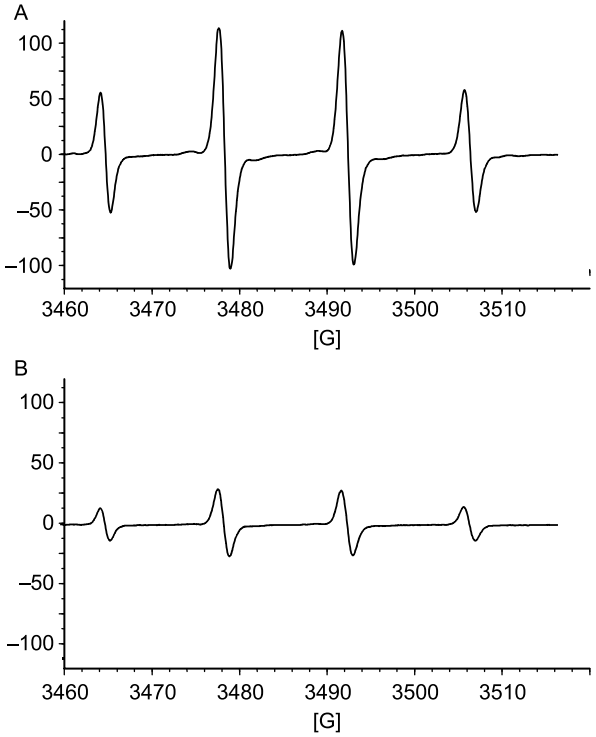

Figure 4. DMPO-OH signal of control (A) and ATP (B). After addition of milliQ (control) or ATP, DMPO-OH spin adducts were analyzed using ESR spectrometry.

incubating with either ADP or ATP. ATP was slightly more effective than ADP in preventing DMPO-OH formation. The DMPO-OH spectrum generated by the Fenton reaction after addition of milliQ (control, spectrum A) or ATP (spectrum B) is shown in Figure 4. Adenosine (5 mM) showed no effect on DMPO-OH spin adduct formation (data not shown). AMP showed a reduction of DMPO-OH spin adduct formation at 3 and 10 mM, but at lower concentrations (0.1, 0.3 and 1 mM) there was no effect (data not shown).

Discussion

We recently [19] reported that ATP resulted in an inhibition of TNF-α and stimulation of IL-10 release in LPS-PHA stimulated whole blood. In the present study, we investigated the anti-inflammatory effects of ATP under different conditions of oxidative stress in stimulated whole blood, a type of experiment not previously performed. We selected the compound ATP because we previously reported that on the one hand, ATP showed a stronger anti-inflammatory effect in stimulated whole blood in comparison with its breakdown product ADP and on the other hand, this effect seemed to be P2 receptor mediated and did not involve the breakdown product adenosine [19]. The rationale underlying the present study was the notion that many inflammatory diseases are characterized by an oxidant/antioxidant imbalance, leading

to oxidative stress [4,10,21]. It is well known that oxidative stress affects both receptor function and signal transmission systems. Thus, Van der Vliet et al. [20] reported that oxidative stress can affect receptor function by influencing the binding of ligands to membrane receptors or the signal transduction system, i.e. coupling of these receptors to G-proteins or affecting the second messenger activity. These effects of oxidative stress could be caused either by peroxidation of membrane lipids, or by interaction of ROS with functional thiol/disulfide groups in the receptor. We therefore, hypothesized that oxidative stress could compromise the previously demonstrated anti-inflammatory properties of ATP under circumstances of oxidative stress. Remarkably, results show that ATP strongly inhibited cytokine release even under circumstances of severe oxidative stress, induced by H_2O_2 at levels as high as 10 mM. These high H_2O_2 concentrations are not directly linked to the *in vivo* situation, but our goal was to investigate a state of extreme oxidative stress. Moreover, it should be noted that catalase, which brakes down most of the H_2O_2, is present in blood and, therefore, the final H_2O_2 concentration in blood will be lower.

The effect of H_2O_2 as an oxidative stress factor on cytokine production has been studied in isolated cells *in vitro*. Indeed, previous studies have shown that H_2O_2 can induce cytokine production in various cell types, such as macrophages [22], cardiomyocytes [23] and keratinocytes [24]. Furthermore, several potential mechanisms underlying H_2O_2-induced cytokine production have been proposed, including NFκB activation [25,26]. We showed that in the absence of LPS and PHA, H_2O_2 induced a dose-dependent increase in the release of TNF-α, IL-10 and IL-6 in whole blood in six out of eight subjects, indicating that the addition of H_2O_2 in this whole blood system induces oxidative stress and thereby activates pro-inflammatory genes. Due to the high inter-individual variation, possibly by a different genetic background of different subjects, the average effect was, however, not significantly different from the control.

H_2O_2, in the absence of ATP, did not alter the LPS-PHA-induced IL-10 or IL-6 production from whole blood relative to LPS-PHA stimulation without H_2O_2. In contrary, at the highest concentration of 10 mM H_2O_2, there was a significant attenuation of LPS-PHA induced TNF-α release. This effect could be caused by interference of H_2O_2 with the LPS mechanism, which is responsible for the TNF-α production by NFκB activation [27,28]. One intriguing explanation for the attenuation of the LPS-PHA-induced TNF-α release in the presence of the highest concentration of H_2O_2, could be that H_2O_2 affects the physical approximation of toll-like receptor-4 (TLR4) with CD14 receptors, an event which is involved in the LPS-induced NFκB activation with subsequent cytokine release [29].

The marked anti-inflammatory effect of ATP, even during severe oxidative stress, might be explained by the possibility that ATP, or some of its breakdown products, may interfere with processes which are directly induced by H_2O_2, e.g. possible protection by ATP against oxidative stress-induced damage at the receptor level, or by influencing ˙OH formation from H_2O_2. We tested whether ATP is able to inhibit the formation of ˙OH, as a potential contribution to its marked effects on cytokine production in the presence of H_2O_2. Results demonstrate that ATP and ADP, even at a concentration of 100 μM, showed a concentration dependent inhibition of the formation of DMPO-OH spin adducts. This implies that ATP, besides its anti-inflammatory effects, also has a radical scavenging effect that might contribute to the cytoprotective and anti-inflammatory effects of ATP in the presence of oxidative stress.

In conclusion, we have shown that ATP exerts strong anti-inflammatory actions in stimulated whole blood under conditions of marked H_2O_2-induced oxidative stress. This suggests that the inhibitory effect of ATP on inflammation is relatively insensitive to oxidative stress. In addition, our findings in ESR experiments that ATP strongly inhibits ˙OH formation, suggests that ATP, in addition to its anti-inflammatory effects, also attenuates oxidative stress itself. Thus, our results indicate that ATP is able to function as a strong combined oxidative stress-inhibiting and anti-inflammatory agent.

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