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Lipopolysaccharide alters nucleotidase activities from lymphocytes and serum of rats

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Abstract

ATP exerts a proinflammatory role and induces cytokine release by acting at $P2X_7$ receptors. The product of ATP hydrolysis is the nucleoside adenosine, an important immunomodulator. The main source of extracellular adenosine is the hydrolysis of extracellular ATP by a group of ecto-enzymes: ENTPDase family, NPP family and ecto-5′-nucleotidase. Considering the role of ATP and adenosine in inflammatory processes, we investigated the effect of lipopolysaccharide on ectonucleotidases activities and expression in lymphocytes from mesenteric lymph nodes and serum of rats, in order to better understand the involvement of extracellular nucleotide hydrolysis in an endotoxemia model. We observed significant changes on nucleotidase activities from lymphocytes and serum of rats after in vitro and in vivo exposure to LPS. In vitro results have shown an increase on nucleotide hydrolysis in lymphocytes and a decrease on the enzyme activity of NPP in blood serum. In vivo, we observed an increase on nucleotide hydrolysis in lymphocytes and a decrease in the hydrolysis of all nucleotides tested in blood serum. After 24 and 48 h of LPS treatment, there was a reduction in NTPDase1, 2, 3 and ecto-5′-nucleotidase transcripts. These results suggest that there is a time-dependent enhancement of extracellular nucleotides metabolism in lymphocytes and blood serum after the induction of an endotoxemic model. The changes observed suggest that these enzymes can act in the regulation of extracellular nucleosides and nucleotides in a model able to trigger inflammatory process.

Keywords: LPS; ATP; Adenosine; NTPDase; NPP; 5′-nucleotidase; Endotoxemia

Introduction

Extracellular ribonucleotides, such as ATP and UTP, have been considered as a new class of signaling molecules that might play a role in inflammation. Ribonucleotides are released at sites of inflammation as a result of cell damage (Di Virgilio et al., 2001). Virtually all cell types express surface receptors for these signaling ribonucleotides. Among these nucleotide receptors, the group of P2 nucleotide receptors comprises P2Y G-protein coupled receptors (P2YR) and the P2X receptors (P2XR), which are ligand-gated ion channels (Ralevic and Burnstock, 1998). P2 receptors are involved in cytokine release (Ferrari et al., 2000; Solle et al., 2001), chemotaxis (McCloskey et al., 1999), vasodilation (Marrelli, 2001; Buvinic et al., 2002), apoptosis (Molloy et al., 1994; von Albertini et al., 1998), T-cell activation and proliferation (Baricordi et al., 1999; Harada et al., 2000), and dendritic cell function (Ferrari et al., 2000). There is convincing evidence that ATP exerts a proinflammatory role and induces cytokine release by acting at $P2X_7$ receptors (Solle et al., 2001).

The product of ATP hydrolysis is the nucleoside adenosine, an important signaling molecule. Adenosine acts through four G-protein-coupled adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) (Fredholm et al., 2001). Adenosine receptors mediate their

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signals by heteromeric G-proteins that can either stimulate (Gs) or inhibit (Gi) adenylyl cyclase. Activation of adenosine receptors on myeloid and lymphoid cells has also been shown to modulate inflammation (Haskó and Cronstein, 2004; Sitkovsky et al., 2004). Endogenous adenosine exerts a significant proportion of its anti-inflammatory actions via binding to A_{2A} receptors found on vascular endothelium, epithelium, monocytes/macrophages, neutrophils, mast cells, lymphocytes, platelets and neurons (Sullivan, 2003). Furthermore, the actions of adenosine receptors are viewed as protecting cardiovascular and neuronal tissues from hypoxia or injury, which can increase the local concentration of extracellular adenosine by 10–100-fold (Fredholm et al., 2001).

The main source of extracellular adenosine is the hydrolysis of extracellular ATP by a group of ecto-enzymes: the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family and the ecto-5′-nucleotidase (EC 3.1.3.5) (Zimmermann, 2001). NTPDases have an important role in cell adhesion and in controlling lymphocytes function, including antigen recognition and/or the effectors activation of cytotoxic T cells (Dombrowski et al., 1995). Furthermore, NTPDases play an important role in lymphocytes, since extracellular nucleotides are mediators of immune and non-immune cell function (Dombrowski et al., 1998). Four members of the family are tightly bound to the plasma membrane via two transmembrane domains, and have a large extracellular region with an active site facing the extracellular milieu. NTPDase1, 3 and 8 slightly prefer ATP over ADP by a ratio of 1, 3 and 2, respectively. Meanwhile, NTPDase2 prefers triphosphonucleosides (Bigonnesse et al., 2004; Vorhoff et al., 2005). NTPDase 1 (CD39) was originally identified as an activation marker for B-lymphocytes and subsequently shown to be expressed on subsets of activated NK (natural killer) cells, T-lymphocytes, epidermal Langerhans DC (dendritic cells) and endothelial cells (Maliszewski et al., 1994). CD39 has also been shown to modulate vascular inflammation, cellular proliferation and migration (Goepfert et al., 2000, 2001) and to play a crucial role in the regulation of the ADP-purinoreceptor P2Y1 function (Enjyoji et al., 1999; Schulte am Esch et al., 1999).

E-NPPs have multiple physiological roles, including nucleotide recycling, modulation of purinergic receptor signaling, regulation of extracellular pyrophosphate levels, stimulation of cell motility, and possible roles in regulation of insulin receptor signaling and activity of ecto-kinases (Goding et al., 2003). Ecto-5′-nucleotidase, otherwise known as CD73, is a lymphocyte maturation marker, which is involved in intracellular signaling, lymphocyte proliferation and activation (Airas, 1998; Resta et al., 1998).

Considering the role of ATP and adenosine in inflammatory processes, we investigated the effect of lipopolysaccharide endotoxin from *Escherichia coli* on ectonucleotidases in lymphocytes and serum from adult rats. Furthermore, we evaluate the E-NTPDases and ecto-5′-nucleotidase expression in mesenteric lymph nodes, in order to better understand the involvement of extracellular nucleotide hydrolysis in an endotoxemia model.

Materials and methods

Animals

In all experiments, male Wistar rats of approximately 60–70 days old, weighing around 250 g from our breeding stock were used and housed four to a cage, with water and food ad libitum. The animal house was kept on a 12 h light/dark cycle (lights on at 7:00 am) at a temperature of 23 ± 1 °C. Procedures for the care and use of animals were adopted according to the regulations of Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council).

In vitro experiments

Different concentrations of LPS (from *E. coli*, serotype 0111:B4) (25, 50, 75 and 100 μg/ml) were tested on nucleotidase activities in serum and lymphocytes from mesenteric lymph nodes of naïve rats. LPS was preincubated with reaction medium during 10 min and, immediately after, the enzyme assays were performed.

In vivo experiments

Rats were injected intraperitoneally with either LPS (2 mg/kg body weight) (Spolarics et al., 1996) or saline. The animals were killed 24 and 48 h after injection.

Isolation of blood serum fraction

Blood samples were drawn after decapitation of rats and were soon centrifuged in plastic tubes at 5000 *g* for 5 min at 20 °C. The serum samples obtained were then stored on ice and immediately used in the experiments (Oses et al., 2004).

Isolation of lymphocytes

Mesenteric lymph nodes were removed and passed through a mesh grid in saline 0.9% (Wu et al., 1991). Cells were washed three times with saline, centrifuged at 200 *g* for 10 min. After, cells were centrifuged two times at 200 *g* for 10 min with the same buffer used in the enzyme assays, without divalent cations. The cells were counted with Trypan Blue and only the groups with more of 95% of viability were used for the experiments.

Enzyme assays

Measurement of serum ρ-Nph-5′-TMP hydrolysis

ρ-Nph-5′-TMP hydrolysis was determined essentially as described by Sakura et al. (1998). The reaction mixture containing ρ-Nph-5′-TMP, as a substrate (at the final concentration of 0.5 mM) in 100 mM Tris–HCl, pH 8.9, was incubated with approximately 1.0 mg of serum protein at 37 °C for 5 min in a final volume at 200 μl. The reaction was stopped by the addition of 200 μl of NaOH 0.2 N. The amount

of ρ-nitrophenol was measured at 400 nm using an extinction coefficient of 18.8×10^{-3}/M/cm. In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped. All samples were assayed in duplicate. Enzyme activities were expressed as nanomoles (nmol) of ρ-nitrophenol released per minute per milligram of protein.

Measurement of blood serum ATP, ADP and AMP hydrolyses

ATP and ADP hydrolyses were determined using a modification of the method described by Yegutkin (1997) according to Oses et al. (2004). The reaction mixture containing 3 mM ATP, ADP or AMP as substrate, 112.5 mM Tris–HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37 °C for 40 min in a final volume of 200 µl. The reaction was stopped by the addition of 200 µl of 10% trichloroacetic acid (TCA). The samples were chilled on ice and the amount of inorganic phosphate (Pi) released was measured as described by Chan et al. (1986). In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with TCA. All samples were centrifuged at 5000 *g* for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. All samples were assayed in triplicate.

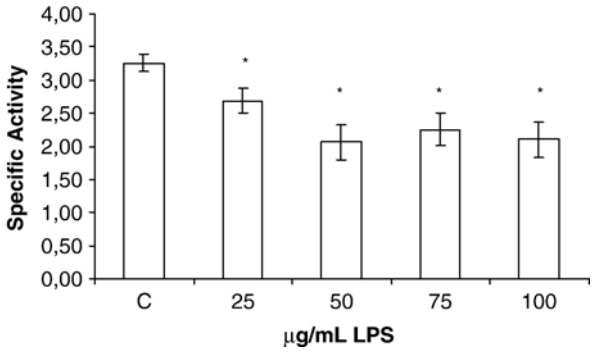

Fig. 2. Influence of different concentrations of LPS (25, 50, 75 and 100 µg/ml) on ρ-Nph-5′-TMP hydrolysis from blood serum fraction of rats. The control of specific activity in serum was 3.3±0.12 nmol ρ-nitrophenol min^{-1} mg^{-1} of protein. The data represent a mean±SD (n=5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P<0.05$ as significant (*).

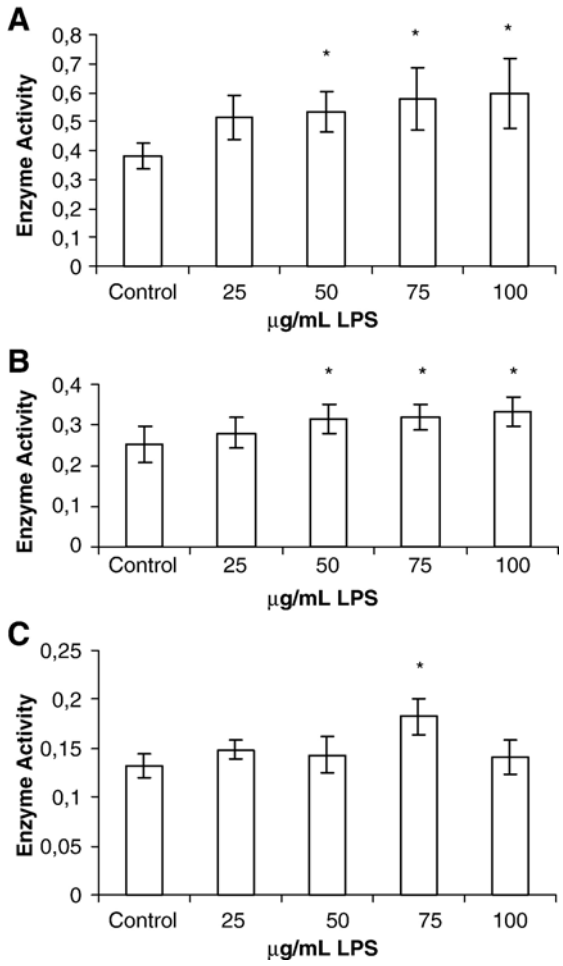

Fig. 1. Influence of different concentrations of LPS (25, 50, 75 and 100 µg/ml) on ATP (A), ADP (B) and AMP (C) hydrolyses in lymphocytes from mesenteric lymph nodes of rats. The control of enzymatic activities in lymphocytes was 0.38±0.05, 0.25±0.04 and 0.13±0.012 nmol Pi $min^{-1} \cdot 10^{-6}$ cells for ATP, ADP and AMP, respectively. The data represent a mean±SD (n=5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P<0.05$ as significant (*).

Measurement of lymphocyte ATP, ADP and AMP hydrolyses

ATP, ADP and AMP hydrolyses were determined using a modification of the method described by Fillippini et al. (1990). The reaction medium contained 2 mM $CaCl_2$ (for ATP and ADP) or $MgCl_2$ (for AMP), 120 mM NaCl, 5 mM KCl, 60 mM glucose, 1 mM sodium azide, 0.1% mM albumin and 20 mM Hepes buffer, pH 7.6, in a final volume of 200 µl. About 10^6 cells of lymphocytes were added to the reaction medium and the enzyme reaction was started by the addition of ATP, ADP or AMP to a final concentration of 2 mM and incubated for 30 min at 37 °C. The reaction was stopped by the addition of 200 µl of 10% TCA. The samples were chilled on ice and the amount of Pi released was measured as described by Chan et al. (1986). In order to correct non-enzymatic hydrolysis, we performed controls by adding the cells after reaction was stopped with TCA. All samples were assayed in triplicate.

Protein determination

Protein was measured by the Coomassie Blue method according to Bradford (1976), using bovine serum albumin as standard.

Analysis of gene expression by semi-quantitative RT-PCR

The analysis of the expression of NTPDases1, 2, 3, 8 and 5′-nucleotidase was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. After 24 h or 48 h of the LPS injection, mesenteric lymph

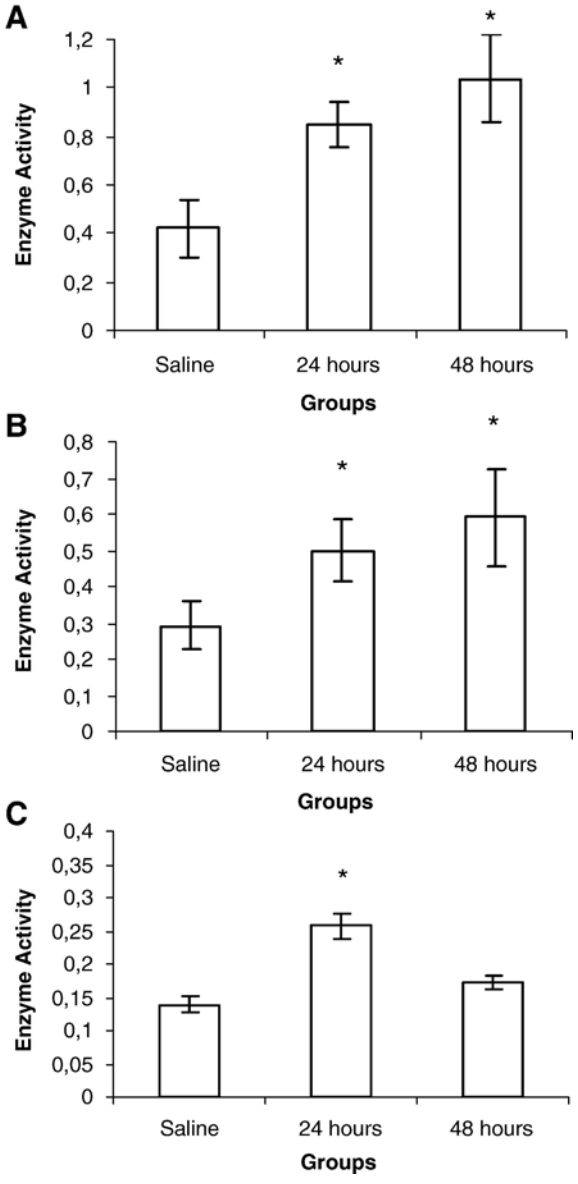

Fig. 3. ATP (A), ADP (B) and AMP (C) hydrolyses in lymphocytes from mesenteric lymph nodes of rats after 24 and 48 h of endotoxemia induction. The control of enzymatic activities in lymphocytes was 0.42±0.12, 0.29±0.06 and 0.14±0.013 nmol Pi $min^{-1} \cdot 10^{-6}$ cells for ATP, ADP and AMP, respectively. The data represent a mean±SD (n=5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P<0.05$ as significant (*).

nodes of rats were isolated for total RNA extraction with Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. RNA purity was quantified spectrophotometrically and tested by eletrophoresis in a 1.0% agarose gel containing ethidium bromide. The cDNA species were synthesized with SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) from 3 μg of total RNA following suppliers. RT reactions were performed for 50 min at 42 °C. cDNA (1 μL) was used as a template for PCR with specific primers for NTPDase1, 2, 3, 8 and 5′-nucleotidase. β-actin was used for normalization as a constitutive gene. PCR reactions have a volume of 25 μL using a concentration of 0.4 μM of each primer indicated below and 200 μM and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer.

Conditions for all PCR were as follows: Initial 1 min denaturation step at 94 °C, 1 min at 94°C, 1 min annealing step (NTPDase1, 3, 8 and 5′-nucleotidase: 65 °C; NTPDase2: 66 °C; β-actin: 58.5 °C), 1 min extension step at 72°C for 35 cycles and a 10 min final extension at 72 °C. The amplification products were: NTPDase1 — 543 bp; NTPDase2 — 331 bp; NTPDase3 — 267 bp; NTPDase8 — 394 bp; 5′-nucleotidase — 403 bp; β-actin — 210 bp. For each set of PCR reactions, negative control was included. Seven microliters of the PCR

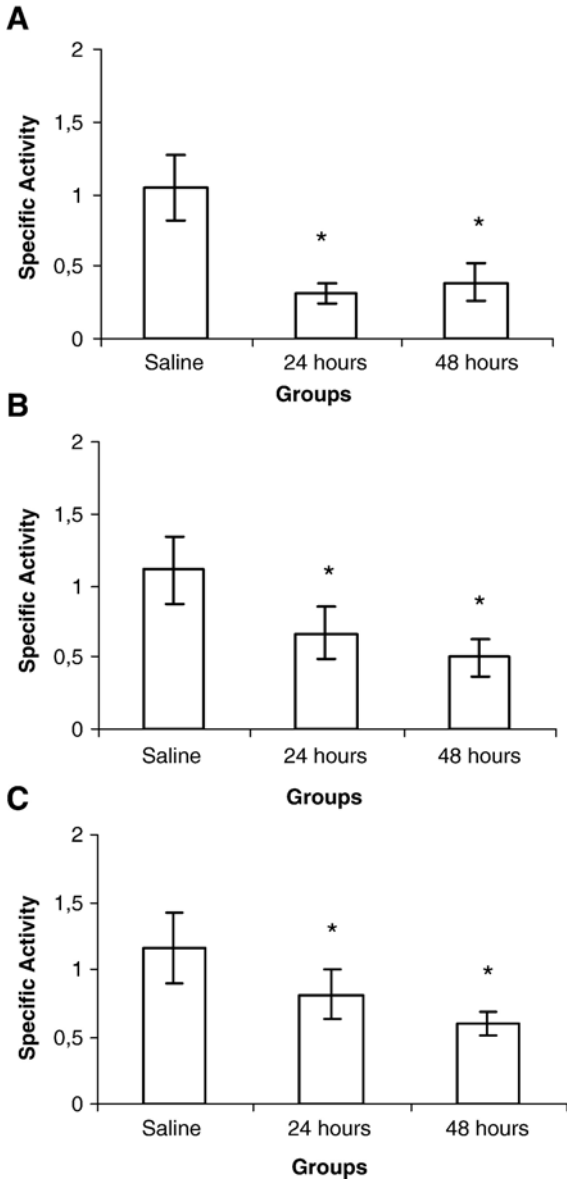

Fig. 4. ATP (A), ADP (B) and AMP (C) hydrolyses from blood serum fraction of rats after 24 and 48 h of endotoxemia induction. The control of specific activities in serum was 1.05±0.23, 1.11±0.23, 1.16±0.26 nmol Pi min^{-1} mg^{-1} of protein for ATP, ADP and AMP, respectively. The data represent a mean±SD (n=5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P<0.05$ as significant (*).

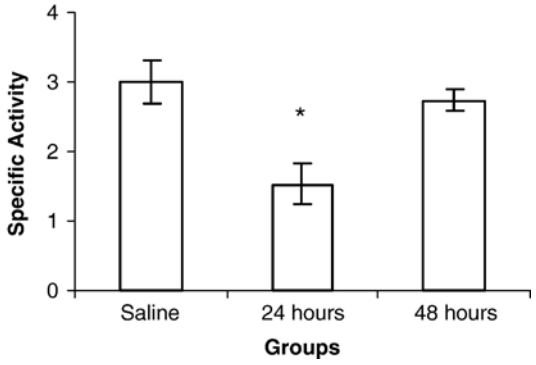

Fig. 5. ρ-Nph-5′-TMP hydrolysis from blood serum fraction of rats after 24 and 48 h of endotoxemia induction. The control of specific activity in serum was 2.99±0.3 nmol ρ-nitrophenol min^{-1} mg^{-1} of protein. The data represent a mean±SD (n=5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P<0.05$ as significant (*).

reaction was analyzed on a 1% agarose gel, containing ethidium bromide and visualized with ultraviolet light. The Low DNA Mass Ladder (Invitrogen) was used as a molecular marker and normalization was performed employing β-actin as a constitutive gene. The following set of primers were used: for NTPDase 1: 5′-GAT CAT CAC TGG GCA GGA GGA AGG-3′ and 5′-AAG ACA CCG TTG AAG GCA CAC TGG-3′; for NTPDase 2: 5′-GCT GGG TGG GCC GGT GGA TAC G-3′ and 5′-ATT GAA GGC CCG GGG ACG CTG AC-3′; for NTPDase 3: 5′-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3′ and 5′-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3′; for NTPDase 8: 5′-CCA CAC TGT CAC TGG CTT CCT TG-3′ and 5′-ACG AGG ATG TAT AGG CCT GAG G-3′; for 5′-nucleotidase (CD73): 5′-CCC GGG GGC CAC TAG CAC CTC A-3′ and 5′-GCC TGG ACC ACG GGA ACC TT-3′; for β-actin: 5′-TAT GCC AAC ACA GTG CTG TCT GG-3′ and 5′-TAC TCC TGC TTC CTG ATC CAC AT-3′.

Statistical analysis

The data obtained are represented as mean±S.D. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Duncan multiple range test, considering $P < 0.05$ as significant.

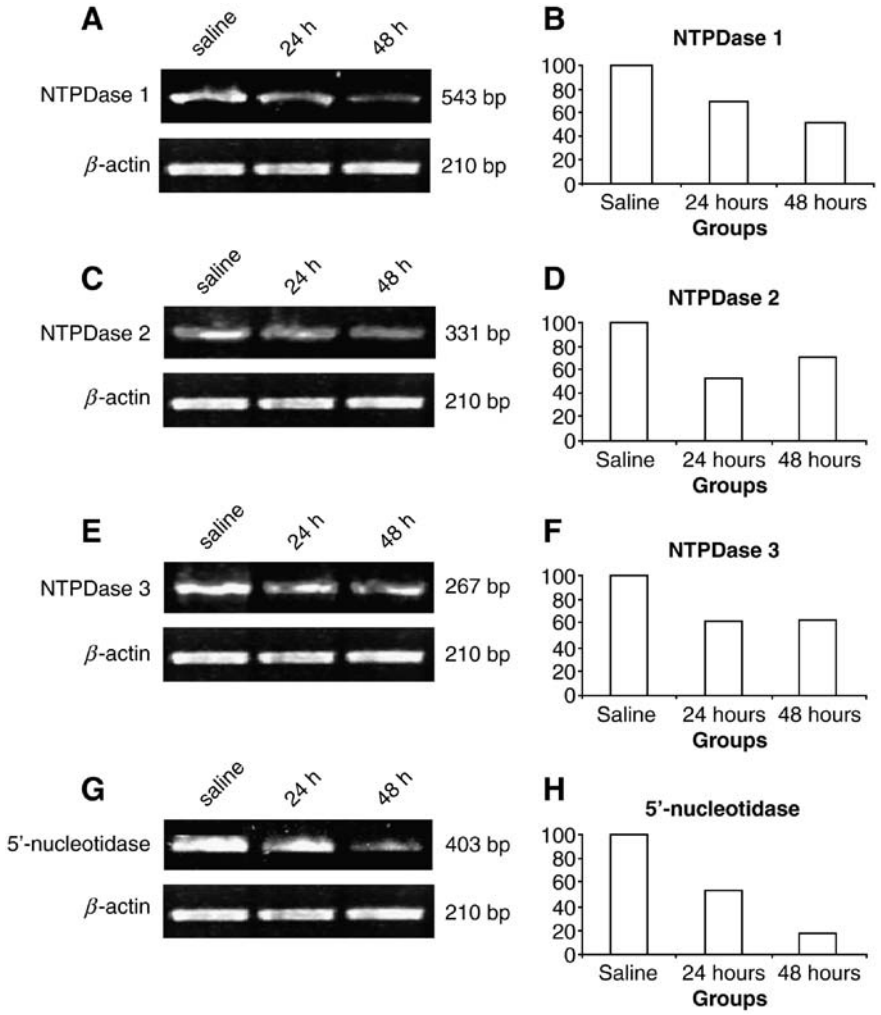

Fig. 6. Gene expression patterns after LPS treatment for NTPDase1 (A and B), NTPDase2 (C and D), NTPDase3 (E and F), 5′-nucleotidase (G and H) and β-actin in mesenteric lymph nodes of rats. Rats were injected with LPS and after 24 and 48 h, the mesenteric lymph nodes were excised and total RNA was isolated being subjected to RT-PCR for the indicated targets. Three independent experiments were performed, with entirely consistent results.

Results

Cellular integrity

The lymphocytes preparation integrity was checked by measuring lymphocytes lactate dehydrogenase (LDH) activity. The protocol was carried out according to the manufacturer's instructions. Triton X-100 (1%, final concentration) was used to disrupt the lymphocyte preparation. The measurement of LDH activity showed that most cells (approximately 90%, $n=3$) were intact after the isolation procedure (data not shown).

In vitro experiments

In lymphocytes, we observed a significant increase on ATP (40%, 50% and 55%) and ADP (24%, 25% and 32%) hydrolyses in the concentration range of 50, 75 and 100 μg/mL, respectively (Fig. 1A and B). AMP hydrolysis just increased significantly at a final concentration of 75 μg/mL (38%) (Fig. 1C). In serum, LPS did not promote a significant difference on ATP, ADP and AMP hydrolyses, when compared to the control (data not shown). The hydrolysis of the artificial substrate ρ-Nph-5′-TMP decreased significantly in the presence of 25, 50, 75 and 100 μg/mL of LPS (18%, 37%, 31% and 36%, respectively), in relation to the control (Fig. 2).

In vivo experiments

In lymphocytes, ATP (100% and 145%) and ADP hydrolyses (69% and 100%) increased significantly at the time tested (24 and 48 h) (Fig. 3A and B). However, AMP hydrolysis showed a pattern similar to the in vitro results, since the hydrolysis of this nucleotide increased significantly at 24 h (92%) after LPS injection and decreased after 48 h of treatment (Fig. 3C). In serum, at 24 and 48 h, we observed a significant decrease in the hydrolysis of the three nucleotide tested (69% and 63% for ATP, 40% and 56% for ADP and 30% and 48% for AMP, respectively) (Fig. 4A, B, and C). The hydrolysis of the artificial substrate ρ-Nph-5′-TMP decreased significantly at 24 h (49%), but it returned to the control levels at 48 h (Fig. 5).

E-NTPDases and ecto-5′-nucleotidase expression in mesenteric lymph nodes

The expression patterns after LPS treatment were represented (Fig. 6). The results have shown that E-NTPDase1, 2, 3 and ecto-5′-nucleotidase transcription were decreased in mesenteric lymph nodes after 24 and 48 h of LPS exposure. We evaluate the E-NTPDase8 transcripts in liver (as positive control) and in mesenteric lymph nodes. However, the results have shown that NTPDase8 is not expressed in this cell type (data not shown).

Discussion

In the present study, we observed significant changes on nucleotidase activities from lymphocytes and serum of rats after in vitro and in vivo exposure to LPS.

ATP and its metabolites, ADP and adenosine, at low concentrations (in the micromolar range) influence vascular tone, cardiac function, platelet aggregation and the function of lymphocytes and granulocytes (Ralevic and Burnstock, 1998; Burnstock, 2004). It has been described an increase of ATP release during inflammation and this compound presents proinflammatory properties (Bodin and Burnstock, 1998). Previous studies have suggested a role for extracellular nucleotides in regulating cellular responses to lipopolysaccharide (LPS). For instance, extracellular signal regulated kinase (ERK) activation by LPS in macrophages can be inhibited by P2 nucleotide antagonists (Hu et al., 1998). Moreover, LPS was shown to activate IL-1 secretion via ATP release and autocrine stimulation (Ferrari et al., 1997; Imai et al., 2000).

ATP is hydrolyzed to ADP, AMP and adenosine by the action of ectonucleotidases (NTPDase family, NPP family and 5′-nucleotidase) (Zimmermann, 2001). In our results, we observed an increase on nucleotide hydrolysis in lymphocytes exposed to different concentrations of LPS, suggesting that this endotoxin can modulate the nucleotide degradation in these cells. The results have shown a significant increase of ATP, ADP and AMP hydrolyses at 24 h after the induction of endotoxemic model. Therefore, an increase of nucleotide hydrolysis could be related to a compensatory response, decreasing ATP availability, a proinflammatory agent and, consequently, contributing to the production of extracellular adenosine, an anti-inflammatory compound. However, at 48 h after the treatment, there is a significant increase of ATP and ADP hydrolyses, but not in AMP hydrolysis, which returns to the control values. Despite these effects, it is possible to suggest that the adenosine levels remain enhanced, due to the stoichiometric effect promoted by the increased ATP and ADP hydrolyses observed. Furthermore, the efficient removal of these nucleotides reduces the ATP/ADP feed-forward inhibition on ecto-5′-nucleotidase, which could allow a burst-like formation of adenosine possibly designed to activate facilitatory A_{2A} receptors (Cunha, 2001).

Several studies have shown the high adenosine levels during inflammatory events or sepsis (Quinlan et al., 1997; Jabs et al., 1998; Martin et al., 2000; Rodriguez-Nunez et al, 2001). It has been reported the role of this purine nucleoside in the control of inflammation, due to its anti-inflammatory properties, acting mainly in adenosine A_{2A} receptors (Sullivan, 2003; Thiel et al., 2003; Capecchi et al., 2005). It has been proposed the administration of adenosine A_{2A} agonists in the inflammatory events and sepsis (Thiel et al., 2003; Sullivan et al., 2004). Selective A_{2A} receptors agonists reduce the extravasation of neutrophils into LPS-challenged tissues in animal models of gram-negative bacterial meningitis (Sullivan at al., 1999) and septic arthritis (Hogan et al., 2001). A_{2A} receptor agonists decrease the serum concentration of TNF in LPS-challenged mice and inhibit LPS-induced release of IL-12 (Haskó et al, 1996) and TNF from isolated mouse macrophages (Haskó et al., 2000). The control of nucleotide and nucleoside levels exerted by ectonucleotidases could contribute to the modulation of purinergic signaling, promoted by P1 and P2 receptors, during inflammatory events.

The alterations promoted by LPS in ecto-nucleotidase activities could be a consequence of transcriptional control. Despite the enzyme activities increased in lymphocytes from mesenteric lymph nodes after LPS exposure, the levels of the E-NTPDase1, 2, 3 and ecto-5′-nucleotidase mRNAs have been decreased. The mechanism that could explain the up-regulation of enzyme activities and at the same time down-regulation of transcriptional levels is known as negative feedback autoregulatory loop. This mechanism allows for genes that are not transcription factors to negatively regulate their own synthesis (Krishna et al., 2006).

Furthermore, we analyzed the soluble nucleotidases from blood serum after in vivo exposure of LPS, since these enzymes could control the circulating nucleotide levels and present an important role in the maintenance of normal physiology (Oses et al., 2004). We observed a decrease on nucleotide hydrolysis after 24 and 48 h of exposure to LPS. The source of soluble NTPDases is unclear, but is possible to suggest the cleavage near the N-terminal region, which result in the release of these enzymes. Our results lead us to the hypothesis that there is a requirement of ectonucleotidases to the cells in order to avoid the harmful effects of ATP. Thus, the cleavage and release of these enzymes could be reduced, promoting a decrease on nucleotide hydrolysis from blood serum.

In summary, these results indicate that there is a time-dependent enhancement of extracellular nucleotides metabolism in lymphocytes and blood serum after the induction of an endotoxemic model. The changes observed suggest that these enzymes can act in the regulation of extracellular nucleosides and nucleotides in a model able to trigger inflammatory process.

Acknowledgements

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