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Endotoxin-induced effects on nucleotide catabolism in mouse kidney

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ABSTRACT

Extracellular adenosine 5′-triphosphate (ATP) acts as a proinflammatory mediator. Adenosine, the final product of ATP breakdown, is an anti-inflammatory compound, acting mainly on adenosine A_{2A} receptors. Considering that the kidney is an organ strongly affected during systemic inflammatory responses and that ectonucleotidases are responsible for the control of extracellular nucleotide and nucleoside levels, we examined the endotoxin-induced effects on ectonucleotidases in kidney membranes of mice, and whether CGS-21680 hydrochloride (3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl] purin-2-yl]amino]ethyl]phenyl]propanoic acid), a selective adenosine A_{2A} receptor agonist, antagonizes the lipopolysaccharide (LPS)-induced effects on nucleotide catabolism in kidney. Animals were injected intraperitoneally with 12 mg/kg LPS and/or 0.5 mg/kg CGS-21680 or saline. Nucleotidase activities were determined in kidney membrane preparations and ATP metabolism was measured by high performance liquid chromatography (HPLC) assay. Analysis of ectonucleotidase expression was carried out by semi-quantitative semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Exposure to endotoxemia promoted an increase in ATP and *p*-Nitrophenyl thymidine 5′-monophosphate (*p*-Nph-5′-TMP) hydrolysis, and a decrease in adenosine 5′-monophosphate (AMP) hydrolysis. CGS-21680 treatment failed to reverse these changes. HPLC analysis indicated a decrease in extracellular ATP and adenosine levels in groups treated with LPS and LPS plus CGS-21680. The expression pattern of ectonucleotidases revealed an increase in Entpd3, Enpp2, and Enpp3 mRNA levels after LPS injection. These findings indicate that nucleotide and nucleoside availability in mouse kidney is altered at different stages of endotoxemia, in order to protect the integrity of this organ when exposed to systemic inflammation.

1. Introduction

Inflammation is an adaptive response to injurious stimuli, such as infection and tissue damage (Medzhitov, 2008). Depending on the trigger, the inflammatory response has a different physiological purpose and pathological consequences. Microbial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, activates Toll-like receptor 4 (TLR4) to induce an inflammatory response (Akira et al., 2001; Alving, 1993). There is evidence that sepsis and septic shock are the most important causes of severe acute kidney injury (AKI) in critically ill patients and correspond to 50% or more of cases of AKI in Intensive Care Units (Hoste et al., 2006; Silvester et al., 2001).

Extracellular nucleotides and nucleosides play important roles during the inflammatory process. Extracellular adenosine 5′-triphosphate (ATP) functions as a proinflammatory and immunostimulatory mediator in the microenvironment of damaged cells (Bours et al., 2006). ATP activates members of the P2 receptor family, comprising P2Y G-protein coupled receptors and P2X receptors, which are ligand-gated ion channels (Ralevic and Burnstock, 1998). Adenosine, the final product of ATP breakdown, is a signaling molecule and adenosine receptors are recognized as important molecular transducers in the pathophysiology of inflammation (Blackburn et al., 2009). Adenosine interacts with members of the P1 receptor family, which consists of four G-protein-coupled receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3 receptors) (Fredholm et al., 2001; Klotz, 2000). Activation of these receptors leads to numerous anti-inflammatory events, including inhibition of T-cell activation (Erdmann et al., 2005; Huang et al., 1997) and limiting the production of inflammatory mediators, such as Interleukin (IL) 12, Tumor necrosis

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factor-α (TNF-α) and Interferon gamma (INFγ) (Haskó et al., 2000; Lappas et al., 2005).

Extracellular nucleotide and nucleoside levels are controlled through the action of ectonucleotidases. This group of ectoenzymes is composed of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the ecto-pyrophosphatase/phosphodiesterase (E-NPP) family and ecto-5′-nucleotidase (EC 3.1.3.5) (Zimmermann, 2001). Eight different *ENTPD* genes encode members of the E-NTPDase protein family, with four of the E-NTPDases (E-NTPDase1, 2, 3, and 8) being expressed as cell surface-located enzymes (Bigonnesse et al., 2004; Zimmermann, 2001). The E-NPP family consists of seven structurally-related ectoenzymes; however, only E-NPP1-3 have the ability to hydrolyze nucleotides and is relevant in the context of the purinergic signaling cascade (Goding et al., 2003; Yegutkin, 2008). Ecto-5′-nucleotidase is the enzyme responsible for adenosine 5′-monophosphate (AMP) hydrolysis, generating the nucleoside adenosine (Zimmermann, 1996), and it has also been proposed to have non-enzymatic functions, such as induction of intracellular signaling and mediation of cell–cell and cell–matrix adhesions (Airas et al., 1997; Resta et al., 1998).

Considering that the kidney is an organ strongly affected during systemic inflammatory responses and that ectonucleotidases are responsible for the control of extracellular nucleotide and nucleoside levels, which play an important role in inflammatory events, we examined the effects of endotoxemia on ectonucleotidase activities, ATP metabolism and mRNA gene expression of these ecto-enzymes in kidney membranes of mice. The effect of CGS-21680 hydrochloride (3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid), a selective adenosine A_{2A} receptor agonist, on nucleotide catabolism in kidney membranes was also investigated.

2. Materials and methods

2.1. Chemicals

CGS-21680, LPS from *Escherichia coli*, serotype 0111:B4, Coomassie Blue, nucleotides, Malachite Green, Trizma Base, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), EDTA (ethylenediaminetetraacetic acid) and *p*-Nitrophenyl thymidine 5′-monophosphate (*p*-Nph-5′-TMP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trizol® Reagent, Deoxyribonucleotide Triphosphates (dNTPs), oligonucleotides, Taq polymerase, Low DNA Mass Ladder, and SuperScript™ III First-Strand Synthesis SuperMix were purchased from Invitrogen (Carlsbad, CA, USA). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA) and GelRed™ was purchased from Biotium (Hayward, CA, USA). All reagents used were of analytical grade.

2.2. Animals

In all experiments, male F1 mice (approximately 8–10 weeks old, weighing around 50 g) from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS, Porto Alegre, RS, Brazil) 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 guidelines of the Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council) and all efforts were made to minimize the number of animals used in this study and their suffering. This study was approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul (UFRGS) under license number 2006628.

2.3. Experimental protocols

The animals received intraperitoneal (i.p.) injections of saline (0.9%), LPS (12 mg/kg) (Pawlinski et al., 2003), and CGS-21680 (0.5 mg/kg body weight) (Martire et al., 2007), according to the groups described below. All solutions were administered in a volume of 2 μL/kg body weight. Mice were randomly divided in: (i) control group (SAL), which received a single injection of saline, (ii) CGS-21680 group (CGS-21680), which received a single injection of CGS-21680, (iii) LPS group (LPS 24 h), which was subjected to endotoxemia by a single injection of LPS, and (iv) LPS plus CGS-21680 (LPS + CGS-21680 24 h), which was subjected to endotoxemia by a single injection of LPS and received a single injection of CGS-21680 immediately after. In order to evaluate the effect of CGS-21680 when the endotoxemia had already become established, the following groups were analyzed: (v) LPS group (LPS 48 h), which was subjected to endotoxemia by a single injection of LPS and 24 h later received a single injection of saline, and (vi) LPS plus CGS-21680 (LPS + CGS-21680 48 h), which was subjected to endotoxemia by a single injection of LPS and 24 h later received a single injection of CGS-21680. All animals were euthanized by decapitation 24 h after the last injection.

2.4. Preparation of kidney membranes

Kidney membranes were prepared essentially as previously described by Nagy and Delgado-Escueta (1984), with minor modifications. Briefly, about 1.0 g of both right and left kidneys was dissected on ice, washed, and gently homogenized in 10 volumes of a medium containing 0.32 M sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.5 (Medium I), before being centrifuged at 1000 *g* for 10 min. The supernatant was collected and centrifuged again at 12000 *g* for 20 min. The resulting supernatant was discarded and the pellet was resuspended in 1.2 mL of Medium I. An aliquot of 1.0 mL of the crude mitochondrial fraction was mixed with 4.0 mL of 8.5% Percoll solution and layered onto an isoosmotic discontinuous Percoll/sucrose gradient (10%/16%). After centrifugation at 15000 *g* for 20 min, the fractions that banded at the 10%/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The kidney membrane fraction was washed twice with Medium I by centrifugation at 15000 *g* for 20 min to remove the contaminating Percoll. The pellet resulting from this second centrifugation was then resuspended to a final concentration of 0.5–0.8 mg/mL. The membranes were prepared fresh daily and maintained at 0–4 °C throughout the experimental procedure.

2.5. Protein determination

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

2.6. Assays of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5′-nucleotidase activities

For the measurement of ATP hydrolysis in membrane fractions, the reaction mixture employed contained 45 mM Tris–HCl, 5.0 mM KCl, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, 1.5 mM $CaCl_2$, 0.1 mM sodium azide, and 2.0 μg/mL oligomycin, pH 7.5, in a final volume of 200 μL. The adenosine 5′-diphosphate (ADP) hydrolysis was assessed using the same reaction mixture described above, except for the fact that 1.0 mM $CaCl_2$ was included and neither sodium azide nor oligomycin were employed in these assays. The activity of ecto-5′-nucleotidase was determined in a reaction medium containing 100 mM Tris–HCl, 1.0 mM $MgSO_4$, pH 7.5, in a final volume of 200 μL. About 13 μg of kidney membrane protein was added to each tube and these were preincubated for 10 min at 37 °C. The enzyme reactions were started by the addition of nucleotides as substrates in a

final concentration of 1.0 mM (ATP/ADP) or 2.0 mM (AMP). After 10 min of incubation, trichloroacetic acid (TCA) (5%, final concentration) was added to stop the reactions. Incubation times, protein concentrations, reaction mixtures, and substrate concentrations were chosen according to previous studies (Fürstenau et al., 2010; Vieira et al., 2001). The amount of inorganic phosphate (Pi) released was determined using a colorimetric method as previously described by Chan et al. (1986). Controls to correct for non-enzymatic substrate hydrolysis were performed by adding membrane fractions after the reactions had been stopped with TCA. All reactions were performed in triplicate. Enzyme activities were generally expressed as nmol Pi released per min per milligram of protein.

2.7. Assay of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity

The phosphodiesterase activity was assessed using *p*-Nph-5′-TMP, an artificial substrate. Briefly, for the assay of kidney membrane E-NPP activity, the reaction medium containing 50 mM Tris–HCl buffer, 5.0 mM KCl, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 1.5 mM $CaCl_2$, pH 8.9, was preincubated with approximately 13 μg per tube of kidney membrane protein for 10 min at 37 °C in a final volume of 200 μL. The enzyme reaction was started by the addition of *p*-Nph-5′-TMP to a final concentration of 0.5 mM. After 10 min of incubation, 200 μL of 0.2 N sodium hydroxide (NaOH) was added to the medium to stop the reaction. Incubation time and protein concentration were chosen in order to ensure the linearity of the reaction. The amount of *p*-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8×10^{-3}/M/cm. Controls to correct for non-enzymatic substrate hydrolysis were performed by adding kidney membrane preparations after the reaction had been stopped with NaOH. All reactions were performed in triplicate. Enzyme activity was generally expressed as nmol *p*-nitrophenol released per min per milligram of protein (Fürstenau et al., 2010; Sakura et al., 1998).

2.8. Analysis of ATP metabolism by high performance liquid chromatography (HPLC)

Kidney membranes from all groups tested were obtained as previously described. The same reaction mixture and protein concentration were used to analyze the metabolism of ATP. However, the enzymatic reactions were started by the addition of 0.1 mM ATP (final concentration) in a final volume of 200 μL at 37 °C. Aliquots of the samples were collected at different times of incubation (0, 3, 10, 30 and 60 min) with the reactions being stopped on ice. All samples were centrifuged at 14000 *g* for 15 min. Aliquots of 40 μL were applied to a reversed-phase HPLC system using a 25 cm C18 Shimadzu column (Shimadzu, Japan) at 260 nm with a mobile phase containing KH_2PO_4 and 5.0 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol as previously described by Voelter et al. (1980). The peaks of purines (ATP, ADP, AMP, and adenosine) were identified by their retention times and quantified by comparison with standards. Results are expressed as nmoles of the different compounds per 200 μL of reaction mixture for each time of incubation. All incubations were carried out in triplicate and the controls to correct for non-enzymatic hydrolysis of nucleotides were performed by measuring the peaks present for the same reaction medium without membranes.

2.9. Analysis of gene expression by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Analysis of NTPDase1 (Entpd1), 2 (Entpd2), 3 (Entpd3), 8 (Entpd8), NPP1 (Enpp1), 2 (Enpp2), 3 (Enpp3), and 5′-nucleotidase (Nt5e) gene expression was carried out by a RT-PCR assay. Twenty-four and/or 48 h after treatments, both right and left kidneys of mice (n = 3 for each group) were removed for total RNA extraction with the Trizol® Reagent in accordance with the manufacturer's instructions. RNA purity was determined spectrophotometrically and assessed by electrophoresis in a 1.0% agarose gel using GelRed™. The cDNA species were synthesized using SuperScript™ III First-Strand Synthesis SuperMix from 3 μg of total RNA following the supplier's instructions. For PCR assays, 1 μL of cDNA was used as a template and screened with specific primers for Entpd1, 2, 3, 8, Enpp1, 2, 3, and Nt5e. PCR was carried out in a volume of 25 μL using a concentration of 0.2 μM of each primer, 200 μM $MgCl_2$, and 1 U Taq polymerase. The cycling conditions for all PCRs were as follows: Initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step (Entpd1, 2, Enpp1 and Actb: 63 °C; Entpd3, Enpp3, Nt5e,: 62 °C; Entpd8: 64 °C; Enpp2: 61 °C), 1 min extension step at 72 °C. These steps were repeated for 35 cycles. Finally, a 10 min extension step was performed at 72 °C. Primer sequences as well as the amplification products are listed in Table 1. Ten microliters of the PCR reaction mixture was analyzed on a 1% agarose gel using GelRed™ and photographed under UV light. The Low DNA Mass Ladder was used as a molecular marker and normalized employing Actb (β-actin) as a constitutive gene. The images of stained PCR products were analyzed by optical densitometry and semi-quantified (enzyme/Actb mRNA ratios) using the computer software Image J.

2.10. Statistical analysis

Results are expressed as means ± standard error (S.E.M.). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test. Statistically significant differences between groups were considered for a $P<0.05$.

3. Results

3.1. Effect of CGS-21680 and modeled endotoxemia on ectonucleotidase activities in kidney membranes

After 48 h of LPS exposure we observed a significant increase in ATP hydrolysis (48%; $P<0.05$) when compared with the control group. When we administered CGS-21680 24 h after LPS exposure (LPS + CGS-21680 48 h) there was a trend towards an increase in ATP hydrolysis, but it was not significant when compared with the control group. In the groups that received LPS and LPS plus CGS-21680 and were analyzed 24 h later, we did not find any significant difference when compared to the control (Fig. 1A). Fig. 1B shows ADP hydrolysis after endotoxemia induction and CGS-21680 exposure; there was no significant difference between the treated groups and the control groups.

Table 1
PCR primers sequences.

Enzyme		Sequence (5′-3′)
Entpd1	Sense	GGT GGC GTC CTT AAG GAC CCG TGC
Entpd1	Antisense	GGA GCT GTC TGT GAA GTT ATA GCC TTG CAG
Entpd2	Sense	CCA CTG TCA GCC TGT CAG GGA CCA GC
Entpd2	Antisense	CGA CAG CCG TGT CTG CCG CCT TC
Entpd3	Sense	ACC GCC TTC ACC TTG GGC CAT G
Entpd3	Antisense	GCT GAG AAG CAG TAG GAC CGG GCA TAC
Enpp1	Sense	TAT TGG CTA TGG ACC TGC CTT CAA GC
Enpp1	Antisense	GTA GAA TCC GGG GCC TCC CGT AG
Enpp2	Sense	GCG ATC TCC TAG GCT TGA AGC CAG C
Enpp2	Antisense	GCT CTG GGA TGC TAG AGA CCT CAG CCT G
Enpp3	Sense	ACA TGC AGG AGA GTT GTC AAC CCC TGC
Enpp3	Antisense	AGA ACA GTG TAT GAA CTC CAC ATG GGC ATC
Nt5e	Sense	CCA TCA CCT GGG AGA ACC TGG CTG C
Nt5e	Antisense	CTT GAT CCG CCC TTC AAC GGC TG
Actb	Sense	GTG CTA TGT TGC TCT AGA CTT CGA GCA GG
Actb	Antisense	CAC CGA TCC ACA CAG AGT ACT TGC GCT C

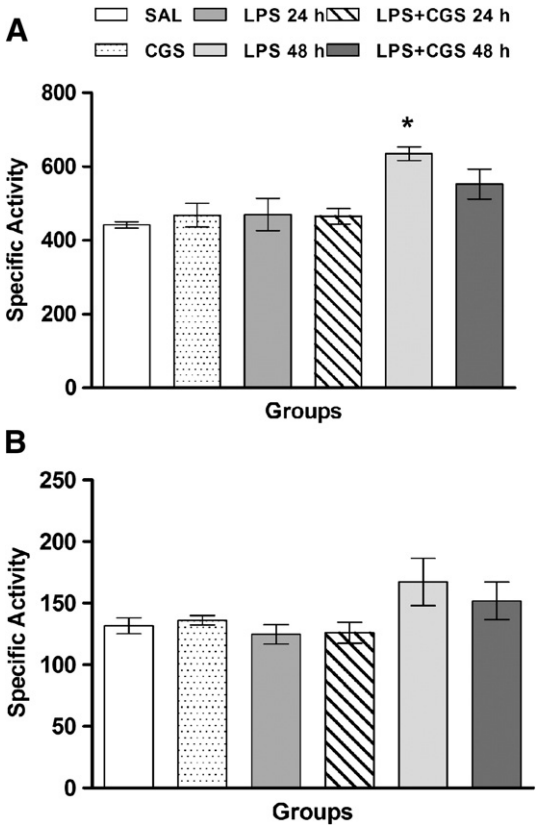

Fig. 1. ATP (A) and ADP (B) hydrolysis in kidney membrane preparations from mice 24 and 48 h after endotoxemia induction and CGS-21680 treatment. The data represent mean ± S.E.M. (n = 5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test, considering $P<0.05$ as significant (*). Mice were divided in: (i) control group (SAL), which received a single injection of saline, (ii) CGS-21680 group (CGS-21680), which received a single injection of CGS-21680, (iii) LPS group (LPS 24 h), which was given a single injection of LPS, (iv) LPS plus CGS-21680 (LPS + CGS-21680 24 h), which was given a single injection of LPS and received a single injection of CGS-21680 immediately after, (v) LPS group (LPS 48 h), which was given a single injection of LPS and 24 h later received a single injection of saline, and (vi) LPS plus CGS-21680 (LPS + CGS-21680 48 h), which was given a single injection of LPS and 24 h later received a single injection of CGS-21680.

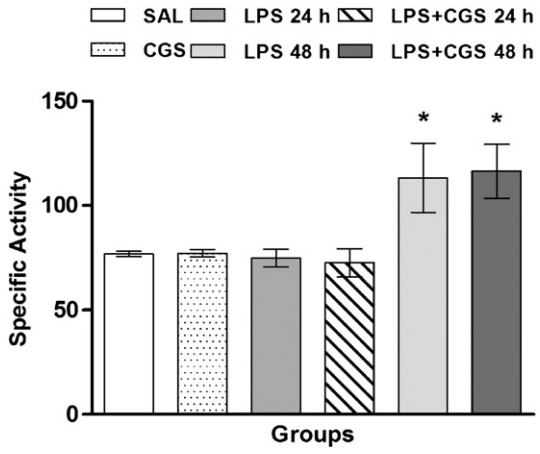

Fig. 2. ρ-Nph-5′-TMP hydrolysis in kidney membrane preparations from mice 24 and 48 h after endotoxemia induction and CGS-21680 treatment. The data represent means ± S.E.M. (n = 5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test, considering $P<0.05$ as significant (*). Mice were divided in: (i) control group (SAL), which received a single injection of saline, (ii) CGS-21680 group (CGS-21680), which received a single injection of CGS-21680, (iii) LPS group (LPS 24 h), which was given a single injection of LPS, (iv) LPS plus CGS-21680 (LPS + CGS-21680 24 h), which was given a single injection of LPS and received a single injection of CGS-21680 immediately after, (v) LPS group (LPS 48 h), which was given a single injection of LPS and 24 h later received a single injection of saline, and (vi) LPS plus CGS-21680 (LPS + CGS-21680 48 h), which was given a single injection of LPS and 24 h later received a single injection of CGS-21680.

The hydrolysis of the artificial substrate, ρ-Nph-5′-TMP, used to determine E-NPP activities, was significantly increased after LPS exposure in LPS 48 h (47%; $P<0.05$) and LPS plus CGS-21680 48 h (51%; $P<0.05$) groups when compared to the saline-control group. This increase was not observed in LPS 24 h and LPS plus CGS-21680 24 h groups (Fig. 2).

For AMP hydrolysis, we observed a significant decrease in the LPS 24 h group (40%; $P<0.05$) and with LPS and CGS-21680 co-administration (37%; $P<0.05$), when compared to the control group. In the LPS 48 h and LPS plus CGS-21680 48 h groups there were no significant differences in AMP hydrolysis when compared with control (Fig. 3). When we injected CGS-21680 alone, it did not alter enzymatic activities for any of the nucleotidases tested compared to saline.

3.2. Metabolism of ATP in kidney membranes of mice exposed to LPS and CGS-21680

The ATP metabolism in the kidney membrane preparations from control and treated groups was measured by HPLC and is presented in Fig. 4. The results show that all treated groups presented a decrease in extracellular ATP levels. In Fig. 4E and F, it can be seen that for AMP, between 3 and 30 min of incubation, LPS 24 h, LPS 48 h, and LPS plus CGS-21680 48 h groups exhibited an accumulation in extracellular levels of this nucleotide. After 10 min of incubation, this accumulation was more apparent in LPS 24 h and LPS 48 h groups (Fig. 4C and E). The extracellular levels of adenosine were decreased in LPS 24 h, LPS plus CGS-21680 24 h, LPS 48 h, and LPS plus CGS-21680 48 h groups when compared to the control in the initial points (3 and 10 min).

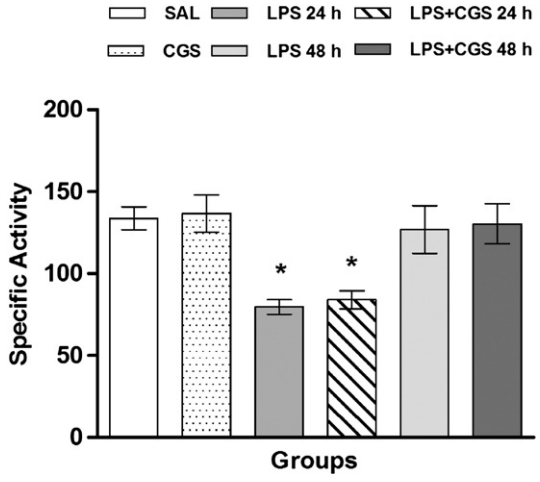

Fig. 3. AMP hydrolysis in kidney membrane preparations from mice 24 and 48 h after endotoxemia induction and CGS-21680 treatment. The data represent means ± S.E.M. (n = 5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test, considering $P<0.05$ as significant (*). Mice were divided in: (i) control group (SAL), which received a single injection of saline, (ii) CGS-21680 group (CGS-21680), which received a single injection of CGS-21680, (iii) LPS group (LPS 24 h), which was given a single injection of LPS, (iv) LPS plus CGS-21680 (LPS + CGS-21680 24 h), which was given a single injection of LPS and received a single injection of CGS-21680 immediately after, (v) LPS group (LPS 48 h), which was given a single injection of LPS and 24 h later received a single injection of saline, and (vi) LPS plus CGS-21680 (LPS + CGS-21680 48 h), which was given a single injection of LPS and 24 h later received a single injection of CGS-21680.

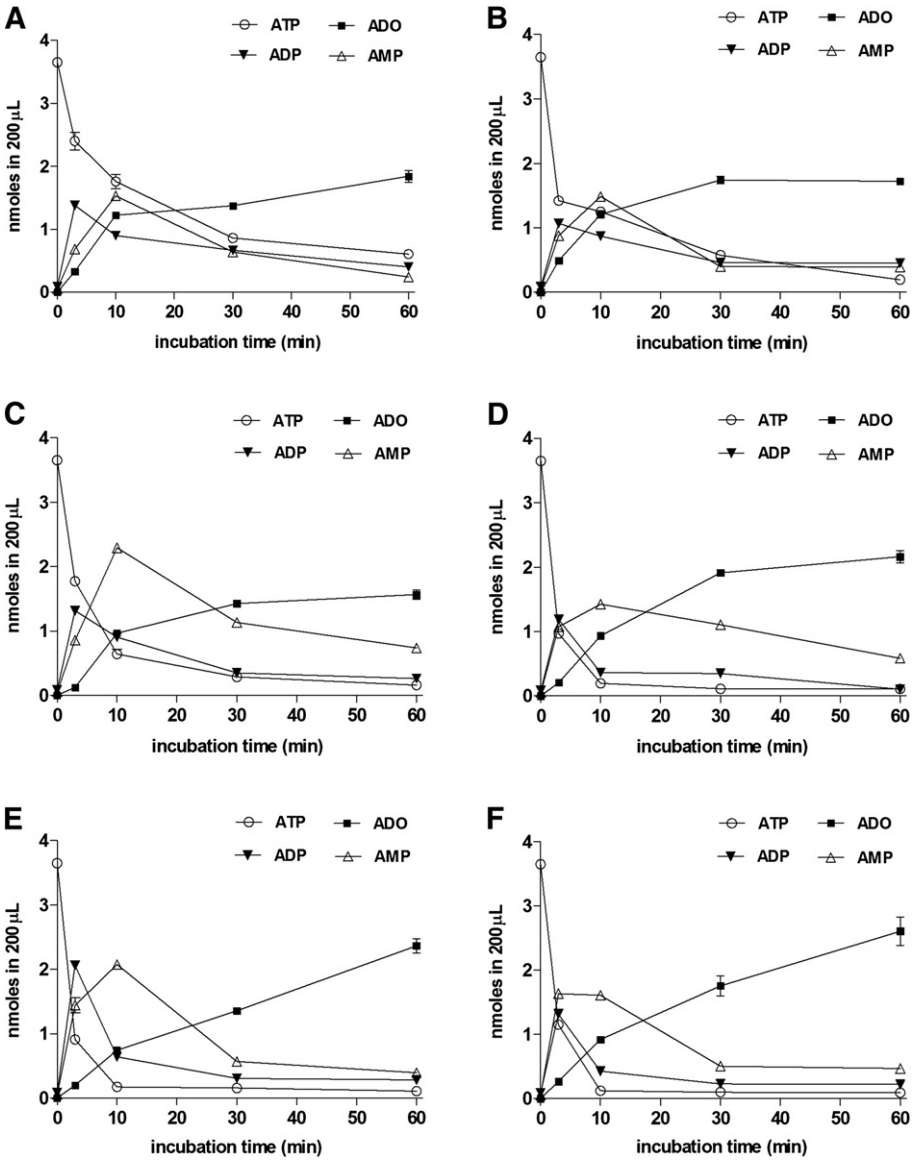

Fig. 4. Metabolism of ATP and product formation in kidney membrane preparations from mice 24 and 48 h after endotoxemia induction and CGS-21680 treatment. Kidney membranes were incubated with 0.1 mM of ATP. The data represent mean ± S.E.M. (n = 3 at least) for (A) control group (SAL), which received a single injection of saline, (B) CGS-21680 group (CGS-21680), which received a single injection of CGS-21680, (C) LPS group (LPS 24 h), which was given a single injection of LPS, (D) LPS plus CGS-21680 (LPS + CGS-21680 24 h), which was given a single injection of LPS and received a single injection of CGS-21680 immediately after, (E) LPS group (LPS 48 h), which was given a single injection of LPS and 24 h later received a single injection of saline, and (F) LPS plus CGS-21680 (LPS + CGS-21680 48 h), which was given a single injection of LPS and 24 h later received a single injection of CGS-21680. ADO = adenosine.

3.3. Effect of CGS-21680 and LPS on ectonucleotidase mRNA expression in kidney

The gene expression patterns of ectonucleotidases after CGS-21680 and LPS treatment were examined and the results showed that 48 h of modeled endotoxemia promoted an increase in Entpd3, Enpp2, and Enpp3 transcript levels. Likewise, the groups that received LPS plus CGS-21680 injection and were subjected to 48 h of endotoxemia (LPS + CGS-21680 48 h) exhibited an increase in the expression of Entpd3, Enpp2, and Enpp3 (Fig. 5). For Nt5e, we did not observe any changes in the levels of mRNA transcripts for the groups tested (data not shown).

4. Discussion

The involvement of extracellular ATP in inflammation has been known since the 1970s and 1980s (Cockcroft and Gomperts, 1980; Dahlquist and Diamant, 1970). This molecule appears to be an "endogenous signal" and exhibits chemotactic and activating effects on leukocytes, which represents potent immuno-enhancing activity (Oppenheim and Yang, 2005). ATP induces the production of inflammatory cytokines, such as IL-1β, IL-2, IL-12, IL-18 and TNFα, via activation of P2X7 receptors, and several pieces of work have demonstrated the participation of ATP in the inflammatory process, acting via this purinoceptor subtype (Elliott and Higgins, 2004; Labasi et al., 2002; Le Feuvre et al., 2002; Solle et al., 2001). Acceptance of the idea that extracellular ATP participates in inflammation increased after P2 receptors were cloned and their expression in inflammatory cells was completely characterized (Di Virgilio et al., 2009). P2 receptors are expressed in the kidney where they are found mainly in the glomeruli and tubules (Bailey et al., 2004; Unwin et al., 2003). Therefore, in the present study, we observed that LPS-induced endotoxemia increased the nucleotide hydrolysis due to E-NTPDase, E-NPP and ecto-5′-nucleotidase activities in kidney membrane preparations from mice, as well as the mRNA transcript levels of these ecto-enzymes.

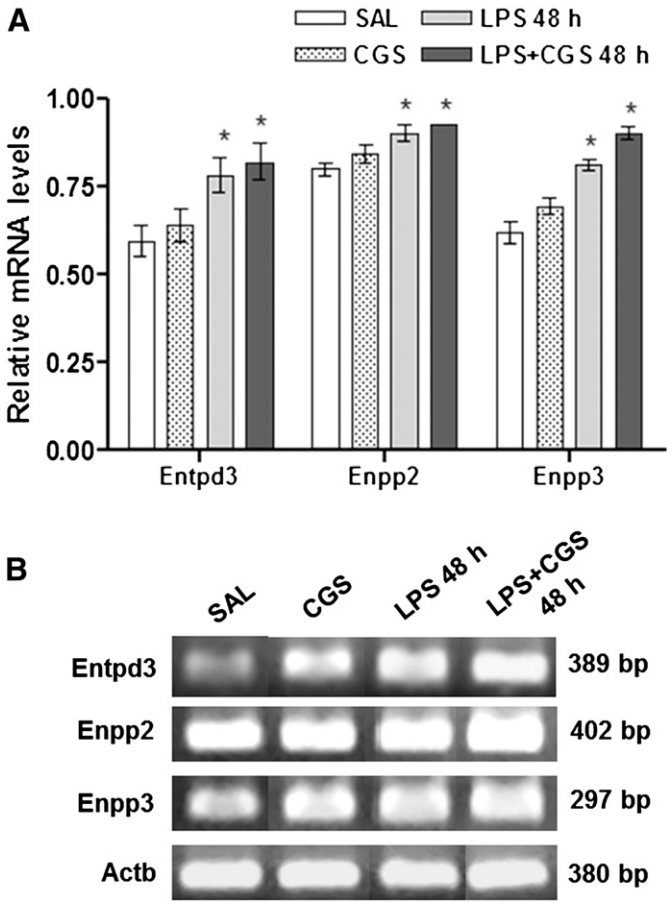

Fig. 5. Gene expression patterns of Entpd3, Enpp2, Enpp3, and Actb in mouse kidney. Total RNA was isolated and subjected to RT-PCR for the indicated targets. Three independent experiments were performed, with entirely consistent results. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test, considering $P<0.05$ as significant (*) difference to control group. Mice were divided in: (i) control group (SAL), which received a single injection of saline, (ii) CGS-21680 group (CGS-21680), which received a single injection of CGS-21680, (iii) LPS group (LPS 24 h), which was given a single injection of LPS, (iv) LPS plus CGS-21680 (LPS+ CGS-21680 24 h), which was given a single injection of LPS and received a single injection of CGS-21680 immediately after, (v) LPS group (LPS 48 h), which was given a single injection of LPS and 24 h later received a single injection of saline, and (vi) LPS plus CGS-21680 (LPS+ CGS-21680 48 h), which was given a single injection of LPS and 24 h later received a single injection of CGS-21680.

ATP is hydrolyzed to ADP, AMP and adenosine by the action of ectonucleotidases (E-NTPDase family, E-NPP family and 5′-nucleotidase) (Zimmermann, 2001). In our results, we observed a significant increase in ATP hydrolysis in kidney membrane preparations after mice had been subjected to 48 h of endotoxemia. For ADP hydrolysis, there was a trend towards an increase in the same group, but it was not statistically significant. As observed for ATP, the hydrolysis of *p*-Nph-5′-TMP, an artificial substrate used to determine NPP activity, was also significantly increased after this time of LPS exposure. These results could be related to a compensatory response 48 h after LPS injection, leading to a decrease in ATP availability, and therefore reducing its proinflammatory influence. Similar results for ATP and ADP hydrolysis were observed by our group when we tested whether LPS injection was able to alter nucleotidase activities in rat lymphocytes from mesenteric lymph nodes (Vuaden et al., 2007). On the other hand, we observed a different pattern of variation for AMP hydrolysis in kidney membrane preparations. We found a significant decrease in AMP hydrolysis 24 h after LPS injection and there was no difference in AMP hydrolysis 48 h after LPS injection compared to the control group. It is possible that AMP hydrolysis 48 h after LPS injection returned to control values due to the stoichiometric effect of the increase in hydrolysis of the other nucleotides. Furthermore, the efficient removal of these nucleotides reduces the ATP/ADP feed-forward inhibition of ecto-5′-nucleotidase, which could allow a burst-like formation of adenosine (Cunha, 2001).

In a similar way to ATP, adenosine can also be considered a molecular danger signal because its extracellular levels rise markedly in response to tissue damage. However, by contrast with ATP, elevated extracellular adenosine levels mediate an autoregulatory immunosuppressive loop to protect healthy tissues (Bours et al., 2006). The four members of the P1 receptor family are all G-protein coupled. A_1 and A_3 receptors are coupled to a Go/Gi protein, causing inhibition of adenylyl cyclase and, consequently, decreasing the production of cAMP (Akbar et al., 1994; Palmer et al., 1995). Conversely, A_{2A} and A_{2B} receptors are coupled to Gs protein, with ligand-binding leading to stimulation of adenylyl cyclase and, in turn, increasing cAMP levels (Olah, 1997; Pierce et al., 1992). A role for adenosine in the control of inflammation has been suggested due to its anti-inflammatory properties, acting mainly on A_{2A} receptors (Capecchi et al., 2005; Sullivan, 2003; Thiel et al., 2003). A_{2A} receptors have taken center stage as the primary anti-inflammatory effectors of extracellular adenosine and have been considered to play a non-redundant role in down-regulating cell-mediated immunity and, also, in activating pro-resolution pathways (Bours et al., 2006; Haskó and Pacher, 2008). A_{2A} receptors in the kidney have been detected in the renal microvasculature (Okusa, 2002), as well as on mesangial (Scholz-Pedretti et al., 2001) and tubular epithelial cells (Lee and Emala, 2002). For this reason, we tested whether CGS-21680, a selective agonist of the adenosine A2A receptor, differently modulates ectonucleotidase activities in kidney membrane preparations from endotoxemic mice. CGS-21680 was administered in two different ways, either concomitant with LPS or 24 h after LPS injection. The changes observed in nucleotide hydrolysis in kidney membrane preparations of mice during modeled endotoxemia were not reversed by CGS-21680 administered concomitantly with LPS, differently for other studies from our group, when we demonstrated that CGS-21680 co-administered with LPS was able to prevent the LPS-induced increase in ATP, AMP, and ρ-Nph-5′-TMP hydrolysis (Vuaden et al., 2011). However, when CGS-21680 was administered 24 h after LPS injection, we observed that the increase in ATP hydrolysis 48 h after LPS exposure was less pronounced if animals were treated with CGS-21680, suggesting that when the LPS-induced inflammatory process has been already established, CGS-21680 was more effective to reverse ATP hydrolysis to control levels. In contrast, for other nucleotide analysis, when LPS was administered 24 h before CGS-21680, this compound failed to reverse the effect of LPS. Since it has been reported that E-NTPDases present different abilities to hydrolyze nucleotides tri and diphosphates (Zimmermann, 2001), we cannot exclude the possibility that LPS might up-regulate the ectonucleotidase activities and that the administration of CGS-21680 could reverse the LPS-induced effect over distinct E-NTPDase members.

Here we report that LPS administration induces changes in ectonucleotidase activities in kidney membrane preparations from mice. The decreased AMP hydrolysis after 24 h of modeled endotoxemia leads us to suggest that, in the initial phase of the inflammatory process, the production of extracellular adenosine via AMP in the kidney was diminished. However, the ATP and *p*-Nph-5′-TMP hydrolysis increased when this process was more established (48 h after LPS injection), indicating that the supply of extracellular ATP is reduced. The analysis of purine metabolism performed by HPLC corroborates this hypothesis, since we observed that ATP levels were markedly decreased in treated groups when compared to control groups. It has been demonstrated that during septic events a possible explanation for the loss of glomerular filtration rate is that although the glomerulus displays both afferent and efferent arteriolar vasodilatation, the efferent vasodilatation is higher (Bellomo et al., 2008). Since adenosine is a vasodilator (Kitakase et al., 1991) and ATP acts as a

proinflammatory agent (Pelegrin et al., 2008), our results lead us to speculate that there is a reduction in adenosine and ATP availability in kidney during inflammatory events in order to protect this organ from the deleterious effects of these molecules.

Purinoceptor and ectonucleotidase expression in immune cells changes under inflammatory conditions, allowing for the progressive acquisition of an immunomodulatory purinergic repertoire by the cells involved in inflammatory and immune responses. The ecto-enzymes ENTPDase1 and ecto-5′-nucleotidase (also identified as CD39 and CD73, respectively) control extracellular nucleotide levels and thus regulate the extent of purinergic signaling (Bours et al., 2006). In order to determine whether the modeled endotoxemia and CGS-21680 could alter ectonucleotidase gene expression, we performed RT-PCR assays. The levels of mRNA transcripts for Entpd3, Enpp2, and Enpp3 were increased after 48 h of LPS exposure, and when CGS-21680 was administered 24 h after LPS. In contrast, we did not observe any changes in Nt5e mRNA levels for the groups tested. For the levels of other enzyme mRNA transcripts analyzed, the differences between treated groups and the control group were not so evident. However, gene expression is regulated by various factors involving cell machinery and signal transduction pathways and enzyme activity cannot be directly correlated with the gene expression pattern or with protein levels due to the existence of several post-translational events (Nedeljkovic et al., 2005). One possible reason for the changes observed in the other ecto-enzymes is the control exerted by phosphorylation, since ectonucleotidases present potential phosphorylation sites on extracellular domains (Bigonnesse et al., 2004; Lavoie et al., 2004).

5. Conclusion

In summary, our results indicate a change in nucleotide and nucleoside viability in the kidneys of mice at different times after endotoxemia induction, in order to protect the integrity of this organ when exposed to inflammation.

Disclosure/Conflict of interest

The authors report no conflicts of interest.

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