European Journal of Pharmacology 651 (2011) 212–217

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and Inflammation

Adenosine A_{2A} receptor agonist (CGS-21680) prevents endotoxin-induced effects on nucleotidase activities in mouse lymphocytes

Fernanda Cenci Vuaden [a], Luiz Eduardo Baggio Savio [a,b], Carolina Maria Alves Bastos [c], Maurício Reis Bogo [b], Carla Denise Bonan [b,*]

[a] *Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 — Anexo, 90035-003, Porto Alegre, RS, Brazil*

[b] *Programa de Pós-Graduação em Biologia Celular e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Caixa Postal 1429, 90619-900, Porto Alegre, RS, Brazil*

[c] *Programa de Pós-Graduação em Farmacologia, Departamento de Farmacologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Três de Maio, 100-Térreo, 04044-020, São Paulo, SP, Brazil*

ARTICLE INFO

Article history:
Received 18 February 2010
Received in revised form 20 October 2010
Accepted 3 November 2010
Available online 27 November 2010

Keywords:
Lipopolysaccharide
Lymphocytes
Nucleoside triphosphate diphosphohydrolase
Nucleotide pyrophosphatase/phosphodiesterase
5′-Nucleotidase
Adenosine

ABSTRACT

Adenosine 5′-triphosphate (ATP) released during inflammation presents proinflammatory properties. Adenosine, produced by catabolism of ATP, is an anti-inflammatory compound. Considering the role of ATP and adenosine in inflammation and the importance of ectonucleotidases in the maintenance of their extracellular levels, we investigated the effect of a selective agonist of the adenosine A_{2A} receptor (CGS-21680) on ectonucleotidase activities and gene expression patterns in lymphocytes from mice submitted to an endotoxemia model. Animals were injected intraperitoneally with 12 mg/kg Lipopolyssacharide (LPS) and/or 0.5 mg/kg CGS-21680 or saline. Nucleotidase activities were determined in lymphocytes from mesenteric lymph nodes and analysis of ectonucleotidase expression was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Exposure to endotoxemia promoted an increase in nucleotide hydrolysis. When CGS-21680 was administered concomitantly with LPS, this increase was prevented for ATP, adenosine 5′-monophosphate (AMP), and p-Nitrophenyl thymidine 5′-monophosphate (p-Nph-5′-TMP) hydrolysis. However, when CGS-21680 was administered 24 h after LPS injection, the increase was not reversed. The expression pattern of ectonucleotidases was not altered between LPS and LPS plus CGS-21680 groups, indicating that the transcriptional control was not involved on the effect exerted for CGS-21680. These results showed an enhancement of extracellular nucleotide catabolism in lymphocytes after induction of endotoxemia, which was prevented, but not reversed by CGS-21680 administration. These findings suggest that the control of nucleotide and nucleoside levels exerted by CGS-21680 could contribute to the modulation of the inflammatory process promoted by adenosine A_{2A} agonists.

1. Introduction

Inflammation is a defense response devised to protect the integrity of the organism against endogenous or exogenous noxious agents (Medzhitov, 2008). The inflammatory response comprises vascular and cellular reactions finely tuned by several mediators, such as chemical factors derived from plasma proteins or cells. Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a molecule that triggers innate immunity and plays an adjuvant effect on adaptive immunity (Condie et al., 1968).

The role of purinergic signaling in the regulation of immune and inflammatory responses has become evident. An increase in ATP release during inflammation has been described and this compound presents proinflammatory properties (Bodin and Burnstock, 1998). Adenosine is a nucleoside formed by the enzymatic breakdown of ATP that acts as neuromodulator in the central and peripheral nervous system. It has been reported that tissue damage and inflammation are accompanied by accumulation of extracellular adenosine due to its release from non-immune and immune cells (Sitkovsky, 2003). Adenosine interacts with four different G-protein-coupled receptors: A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001). Because this nucleoside presents anti-inflammatory properties, acting mainly on adenosine A_{2A} receptors, a role of adenosine in the control of inflammation has been suggested (Sullivan, 2003; Thiel et al., 2003; Capecchi et al., 2006). Furthermore, it has been proposed that the administration of adenosine A_{2A} agonists could be useful in inflammatory events and sepsis (Thiel et al., 2003; Sullivan et al., 2004).

---

[*] Corresponding author. Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Caixa Postal 1429, 90619-900, Porto Alegre, RS, Brazil. Tel.: +55 51 3353 4158; fax: +55 51 3320 3568.
E-mail address: cbonan@pucrs.br (C.D. Bonan).

0014-2999
doi:10.1016/j.ejphar.2010.11.003

The presence of nucleotide-metabolizing pathways on the surface of immune and non-immune cells, which also co-express ATP and adenosine receptors, is essential for the fine-tuning regulation of the duration and magnitude of purinergic signaling. Several enzyme families are involved in the control of nucleotide and nucleoside levels: the ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDase), the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) and the ecto-5′-nucleotidase (EC 3.1.3.5) (Zimmermann, 2001). E-NTPDases have an important role in cell adhesion and in controlling lymphocyte function, including antigen recognition and/or the effector activation of cytotoxic T cells (Dombrowski et al., 1995; Dombrowski et al., 1998). Four members (E-NTPDase1-3 and 8) 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. 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 the regulation of insulin receptor signaling and the activity of ecto-kinases (Goding et al., 2003). Ecto-5′-nucleotidase (CD73) is a lymphocyte maturation marker, which is involved in intracellular signaling, lymphocyte proliferation and activation (Airas, 1998; Resta et al., 1998).

Considering the roles that the nucleotides and nucleosides play during inflammatory events, and the importance of ectonucleotidases for the maintenance of extracellular levels of the former, we investigated the effect of a selective agonist of the adenosine A_{2A} receptor (CGS-21680) on ectonucleotidase activities in lymphocytes from mice submitted to an endotoxemia model. Furthermore, the effect of LPS and CGS-21680 on the E-NTPDase, E-NPP, and ecto-5′-nucleotidase gene expression were also evaluated in mesenteric lymph nodes.

2. Materials and methods

2.1. Chemicals

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), HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid), Lipopolysaccharide (LPS) from *Escherichia coli*, serotype 0111:B4, nucleotides (ATP, ADP, and AMP), Malachite Green, Trizma Base, and *p*-Nitrophenyl thymidine 5′-monophosphate (*p*-Nph-5′-TMP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The RNASpin Ilustra Mini Kit for RNA isolation was purchased from GE Healthcare. 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). The LDH Liquiform Kit was purchased from Labtest Diagnóstica S.A. (Lagoa Santa, MG, Brazil). All reagents 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 regulations 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 saline injection, (ii) CGS group (CGS), which received a single injection of CGS-21680, (iii) LPS group (LPS 24 h), which was submitted to the endotoxemia model by a single injection of LPS, and (iv) LPS + CGS (LPS + CGS 24 h), which was submitted to the endotoxemia model 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 submitted to the endotoxemia model by a single injection of LPS and 24 h later received a single injection of saline, and (vi) LPS + CGS (LPS + CGS 48 h), which was submitted to the endotoxemia model 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. Isolation of lymphocytes

Mesenteric lymph nodes were removed and passed through a mesh grid in wash buffer (the same buffer used in the enzyme assays, without divalent cations). Cells were washed two times with this buffer by centrifugation at 200 g for 10 min. After, the cells were stained with 0.1% Trypan Blue and counted, and only the groups with more than 95% viability were used in the experiments.

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

The reaction medium contained 1 mM $CaCl_2$ (for ATP and ADP hydrolysis) or $MgCl_2$ (for AMP hydrolysis), 120 mM NaCl, 5 mM KCl, 60 mM glucose, 1 mM sodium azide, 0.1% mM albumin, and 20 mM Hepes buffer, pH 7.5, in a final volume of 200 µl. Approximately 10^8 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, followed by incubation for 30 min at 37 °C. The reaction was stopped with 200 µl of 10% trichloroacetic acid (TCA). Incubation times, protein concentrations, reaction mixtures, and substrate concentration were chosen and modified according to a study previously published by Vuaden et al. (2007). The amount of inorganic phosphate (Pi) released was measured using a colorimetric method as previously outlined by Chan et al. (1986). Controls to correct for non-enzymatic substrate hydrolysis were performed by adding the cells 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 10^8 cells.

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

The phosphodiesterase activity was assessed using ρ-Nph-5′-TMP (an artificial substrate). The reaction medium contained 1 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose, 1 mM sodium azide, 0.1% mM albumin, and 20 mM Tris buffer, pH 8.9, in a final volume of 200 µl. Approximately 10^8 lymphocytes were added to the reaction medium and the enzyme reaction was started by the addition of ρ-Nph-5′-TMP to a final concentration of 0.5 mM. After 60 min of incubation, 200 µl of 0.2 N NaOH were 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 ρ-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 the cells after the reaction had been stopped with NaOH. All reactions were performed in triplicate. Enzyme activity was generally expressed as nmol ρ-nitrophenol released per min per 10^8 cells (Sakura et al., 1998; Vuaden et al., 2009).

2.7. Analysis of gene expression by semi-quantitative 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 semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Twenty-four and/or 48 h after treatments, mesenteric lymph nodes of mice (n = 3 for each group) were removed for total RNA extraction with the RNASpin Ilustra Mini Kit in accordance with the manufacturer's instructions. RNA purity was quantified 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 reactions were 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 and Nt5e: 62 °C; Entpd8: 64 °C; Enpp: 61 °C), 1 min extension step at 72 °C. These steps were repeated for 35 cycles. Finally, a 10 min post-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 were 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 normalization was performed 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.

2.8. 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 $P < 0.05$.

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

3. Results

3.1. Cellular integrity

The lymphocyte preparation integrity was checked by measuring lymphocyte lactate dehydrogenase (LDH) activity. The ratio of this enzyme activity measured in intact and disrupted lymphocytes can be regarded as a measure of damaged particles. 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).

3.2. Effect of LPS-induced endotoxemia model and CGS-21680 on ectonucleotidase activities in lymphocytes

After 24 h of LPS exposure we observed a significant increase in ATP hydrolysis (178%) when compared with the control group. This increase was prevented when CGS-21680 was co-administered with LPS. Likewise, after 48 h of LPS exposure, we observed a significant increase in ATP hydrolysis (135%) when compared with control, although this was not reversed by the administration of CGS-21680 (Fig. 1A). Fig. 1B shows that when compared to control ADP hydrolysis increased significantly after induction of endotoxemia with LPS. However, there was no significant difference between groups that received LPS plus CGS-21680 and the groups that received LPS only.

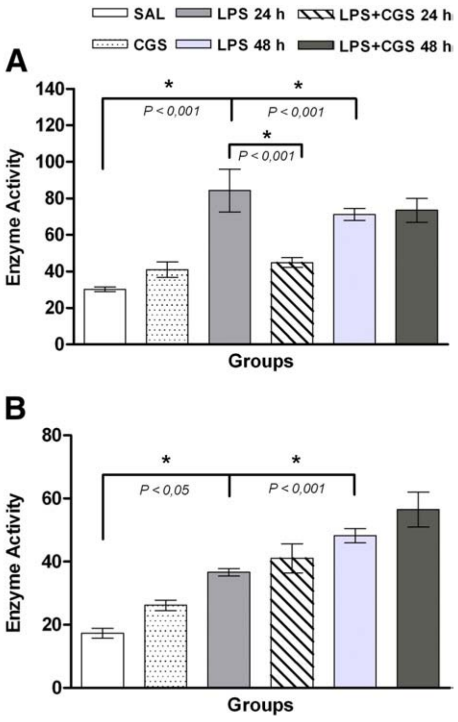

Fig. 1. ATP (A) and ADP (B) hydrolysis in lymphocytes from mesenteric lymph nodes of mice 24 and 48 h after endotoxemia induction and CGS-21680 treatment. The data represent mean ± S.E.M. (n = 5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test, considering $P < 0.05$ as significant (*). Mice were divided in: (i) control group (SAL), which received a single saline injection, (ii) CGS group (CGS), which received a single injection of CGS-21680, (iii) LPS group (LPS 24 h), which was submitted to a single injection of LPS, (iv) LPS + CGS (LPS + CGS 24 h), which was submitted to a single injection of LPS and received a single injection of CGS-21680 immediately after, (v) LPS group (LPS 48 h), which was submitted to a single injection of LPS and 24 h later received a single injection of saline, and (vi) LPS + CGS (LPS + CGS 48 h), which was submitted to 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 phosphodiesterase activities, was significantly increased after 24 h of LPS treatment when compared to control (117%). This increase was prevented by the co-administration of CGS-21680. After 48 h of LPS injection, there was a significant increase (147%) in ρ-Nph-5′-TMP hydrolysis when compared to saline-treated controls (Fig. 2) and this increase persisted after CGS-21680 administration. For AMP hydrolysis we observed a significant increase after 24 h of LPS exposure (180%) when compared to the control group and the hydrolysis of this nucleotide was similar to control group after co-administration of LPS plus CGS-21680. After 48 h of LPS treatment, we verified a significant increase in AMP hydrolysis (105%), and the CGS-21680 administration did not reverse this effect (Fig. 3).

3.3. Effect of LPS and CGS-21680 on ectonucleotidase mRNA expression in mesenteric lymph nodes

The results show that mRNA transcript levels were altered in after LPS and LPS plus CGS-21680 treatments when compared to saline group for Entpd3 and Enpp3 (Fig. 4A and B). For others enzyme mRNA transcripts we did not observe significant alterations in mesenteric lymph nodes after the treatments (data not shown). We examined the Entpd8 transcripts in liver (as a positive control) and in mesenteric lymph nodes. However, we found that Entpd8 was not expressed in lymph nodes (data not shown).

4. Discussion

In the present study, we observed that LPS-induced endotoxemia model increased the nucleotide hydrolysis promoted by E-NTPDases, E-NPP and ecto-5′-nucleotidase activities in lymphocytes of mice. In addition, the co-administration of CGS-21680 with LPS was able to prevent the increase of enzyme activities.

A role for the purinergic system in the immune response has become more accepted over the last few years. In a brief update, Di Virgilio (2007) reported that interest in this hypothesis is slowly growing among the immunological community, as observed by the increase in the number of papers reporting the effect of purinergic agonists on many different immune-mediated responses. It has been demonstrated that extracellular ATP is accumulated at sites of inflammation and induces an inflammatory response, being, in relevant amounts, considered a signal of tissue injury or distress (Di Virgilio et al., 2009). Other work has shown that ATP acts as an immunomodulatory agent via P2X and P2Y receptors, more specifically via the P2X7 subtype (Ferrari et al., 2006). In addition, adenosine, the final product of ATP hydrolysis, exerts anti-inflammatory effects via A_{2A} receptors (Di Virgilio et al., 2009).

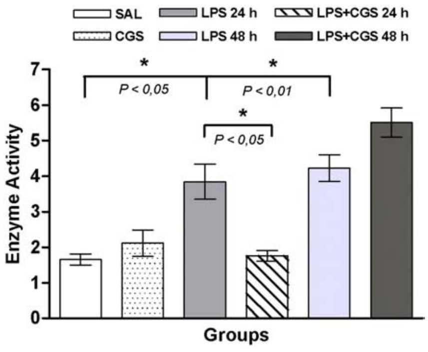

Fig. 2. ρ-Nph-5′-TMP hydrolysis (A) in lymphocytes from mesenteric lymph nodes of mice 24 and 48 h after endotoxemia induction and CGS-21680 treatment. The data represent a mean ± S.E.M. (n = 5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Tukey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

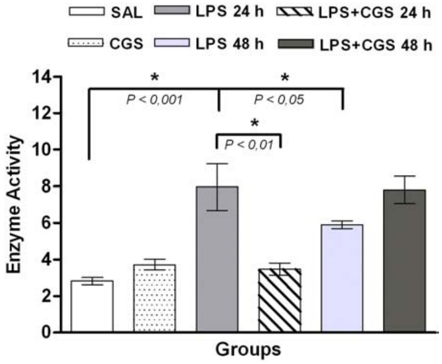

Fig. 3. AMP hydrolysis (A) in lymphocytes from mesenteric lymph nodes of mice 24 and 48 h after endotoxemia induction and CGS-21680 treatment. The data represent a mean ± S.E.M. (n = 5 at least). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple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

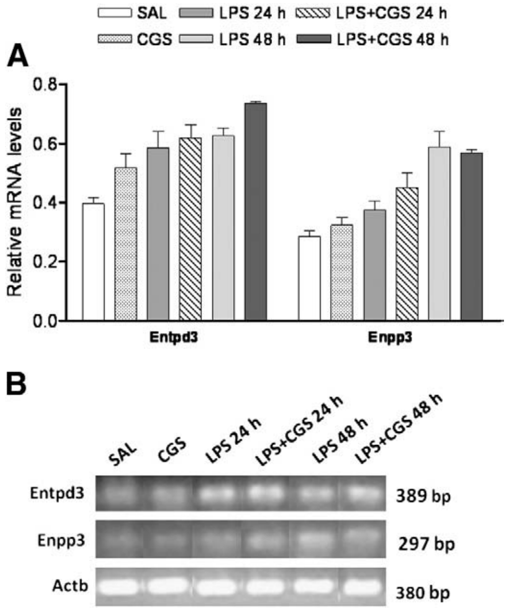

Fig. 4. Gene expression patterns of Entpd3, Enpp3, and Actb in mesenteric lymph nodes of mice. Total RNA was isolated and subjected to RT-PCR for the indicated targets. Three independent experiments were performed, with entirely consistent results. Mice were divided in: (i) control group (SAL), which received a single saline injection, (ii) CGS group (CGS), which received a single injection of CGS-21680, (iii) LPS group (LPS 24 h), which was submitted to a single injection of LPS, (iv) LPS + CGS (LPS + CGS 24 h), which was submitted to a single injection of LPS and received a single injection of CGS-21680 immediately after, (v) LPS group (LPS 48 h), which was submitted to a single injection of LPS and 24 h later received a single injection of saline, and (vi) LPS + CGS (LPS + CGS 48 h), which was submitted to a single injection of LPS and 24 h later received a single injection of CGS-21680.

The enzymes responsible for ATP hydrolysis are named ectonucleotidases (NTPDase family, NPP family and ecto-5′-nucleotidase) (Zimmermann, 2001). Besides their involvement in the role of ATP in inflammation, enzymes that degrade extracellular nucleotides, such as NTPDase1 (CD39) and 5′-nucleotidase (CD73), present immunomodulatory activity (Dwyer et al., 2007). Furthermore, NTPDases play an important role in lymphocyte function, since extracellular nucleotides are mediators of immune and non-immune cell function (Dombrowski et al., 1998).

Our results demonstrated a significant increase in nucleotide hydrolysis in lymphocytes after mice had been exposed to endotoxemia model, both at 24 h and 48 h after LPS exposure. A similar result was observed previously in our laboratory, when rats were injected with LPS and the hydrolysis of ATP, ADP, and AMP in lymphocytes from mesenteric lymph nodes was determined later (Vuaden et al., 2007). This increase in nucleotide hydrolysis could be related to a compensatory response, decreasing the availability of ATP, a proinflammatory agent, and, consequently, contributing to the production of extracellular adenosine, an anti-inflammatory compound. Here we investigated the effect of CGS-21680, a selective A_{2A} adenosine receptor agonist, on nucleotide catabolism. When CGS-21680 was administered alone, we did not observe a significant difference compared to the control group. However, when CGS-21680 was administered concomitantly with LPS, it was able to prevent the LPS-induced increase in ATP, AMP, and ρ-Nph-5′-TMP hydrolysis (LPS + CGS 24 h group). In contrast, when CGS-21680 was administered 24 h after LPS injection, the increase was not reversed (LPS + CGS 48 h group). Therefore, we suggest that this selective A_{2A} agonist can prevent the endotoxemic effects of LPS, but cannot reverse such effects when the endotoxemia model has already been established. Despite the effects observed on ATP hydrolysis, there was no significant difference between LPS and LPS plus GCS-21680 groups for ADP hydrolysis. Since it has been reported that E-NTPDases present different abilities to hydrolyze tri and diphosphonucleosides (Zimmermann, 2001), we cannot exclude the possibility that LPS might up-regulate the ectonucleotidase activities and that the co-administration of CGS-21680 could prevent the LPS-induced effect over distinct E-NTPDase members.

In order to evaluate whether the endotoxemia model and CGS-21680 could alter the ectonucleotidase gene expression, we performed RT-PCR assays. The gene expression pattern of ectonucleotidases presented an increase in mRNA levels in groups treated with LPS and LPS plus CGS-21680 mainly for Entpd3 and Enpp3. For other enzyme mRNA transcript levels analyzed, the differences between treated groups and control group were not so evident. Interestingly, these results differ from the rat model of endotoxemia in which we previously demonstrated that LPS decreased the Entpd and Nt5e mRNA levels from rat lymph nodes (Vuaden et al., 2007). Although LPS also induced an increase on ectonucleotidase activities from rat lymphocytes, the regulation of gene expression is dependent of various aspects involving cell machinery and transduction signaling pathways. Since enzyme activity cannot be directly correlated to gene expression pattern or to protein levels due to the existence of several post-translational events (Nedeljkovic et al., 2005), these apparently discrepancies between rat and mice gene expression profile in the LPS-induced endotoxemia model still require further investigations.

Adenosine receptors are coupled to G-proteins, and adenosine A_{2A} and A_{2B} receptors in particular can increase intracellular cAMP levels by activating adenylate cyclase. The expression pattern of adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) varies depending on the cell type and pharmacological and biochemical studies have established that A_{2A} receptor is the predominant subtype in immune cells (Huang et al., 1997; Koshiba et al., 1999). The anti-inflammatory effects of extracellular adenosine mediated through adenosine receptor signaling have been known and investigated for a long time (Fredholm et al., 2001). Numerous studies in cellular and animal model systems have provided evidence that A_{2A} signaling pathways are active in limiting inflammation and tissue injury (Hasko and Cronstein, 2004; Linden, 2005; Sitkovsky and Ohta, 2005; Hasko and Pacher, 2008). The interaction between adenosine and A_{2A} receptors is capable of inhibiting inflammation by cAMP induction (Ohta and Sitkovsky, 2009). Experimental data suggest that A_{2A} agonists and antagonists can mediate inflammation by activating and blocking, respectively, an A_{2A}-dependent immunomodulatory mechanism (Ohta and Sitkovsky, 2001). A_{2A} receptors has numerous anti-inflammatory properties, as well as inhibiting T-cell activation (Huang et al., 1997; Erdmann et al., 2005) and limiting the production of inflammatory mediators, such as IL-12, TNF-α· and INFγ (Hasko et al., 2000; Pinhal-Enfield et al., 2003; Lappas et al., 2005).

It is important mention that there are some limitations in the current study. Firstly, we have performed these experiments using a single dose of one adenosine A_{2A} agonist. Studies have already shown that other agonists of A_{2A} adenosine receptors present anti-inflammatory actions, such as ATL146e, ATL313, and IB-MECA (Sullivan et al., 2004; Odashima et al., 2005; Lappas et al., 2006; Sevigny et al., 2007). However, few and recent studies have demonstrated that CGS-21680 is able to protect or revert inflammatory states (Lappas et al., 2005; Genovese et al., 2009; Kreckler et al., 2009). The choice of this CGS-21680 dose, which was administered i.p., was base on previous studies indicating its ability to activate adenosine A_{2A} receptor (Martire et al., 2007). We have chosen to test CGS-21680 in two different times of LPS exposures in order to evaluate potential actions of this compound on purine catabolism in modeled endotoxemia. Therefore, testing different CGS-21680 doses and adenosine A_{2A} agonists would reinforce the idea that activation of adenosine A_{2A} receptors may exert a modulatory role in the inflammatory responses. Furthermore, other adenosine A_{2A} antagonists, such as caffeine or ZM241385, can be tested in order to evaluate a possible increase in the severity of the inflammatory state after modeled endotoxemia. We consider that our findings may support future studies aiming the use of different doses of CGS-21680 and different adenosine agonists as anti-inflammatory compounds.

A study performed by Deaglio et al. (2007) provided new information about the mechanism of adenosine generation and immunoregulation by Tregs cells. They demonstrated that Tregs cells express a unique combination of both CD39 (E-NTPDase1) and CD73 (ecto-5′-nucleotidase). These findings demonstrate that the production of adenosine through the enzymatic cascade on the surface of Tregs is important to the A_{2A}-mediated immunosuppressive effects of these cells (Deaglio et al., 2007). These results provide an example of how the coordinate regulation of adenosine production and signaling can impact the immune response. Here we demonstrate that the nucleotide hydrolysis was increased when endotoxemia model was induced in mice. We hypothesized that this increase in ectonucleotidase activities promoted by LPS is a response against the inflammatory process, resulting in ATP depletion and adenosine generation. The effect of LPS on ectonucleotidase activities was prevented when LPS was co-administered with CGS-21680. However, when LPS was administered 24 h before CGS-21680, this compound failed to reverse the effect of LPS. Probably, in the initial phase, when LPS not exert all of inflammatory effects yet, the selective A_{2A} receptor agonist was able to prevent the ectonucleotidase stimulation. In contrast, when the LPS-induced inflammatory process has been already established and the production of adenosine has been enhanced, CGS-21680 was not effective to reverse ectonucleotidase activities to control levels.

5. Conclusion

In summary, our results demonstrate an enhancement of extracellular nucleotide catabolism in lymphocytes after the induction of the modeled endotoxemia, which was prevented, but not reversed by

CGS-21680 administration. These data suggest that the protective effect of CGS-21680 on the LPS-induced alterations in the ectonucleotidase activities could be related, at least in part, to the effects promoted by the A_{2A} receptor agonists during inflammation.

Acknowledgements

This work was supported by grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

References

Airas, L., 1998. CD73 and adhesion of B-cells to follicular dendritic cells. Leuk. Lymphoma 29, 37–47.

Bodin, P., Burnstock, G., 1998. Increased release of ATP from endothelial cells during acute inflammation. Inflamm. Res. 47, 351–354.

Capecchi, P.L., Camurri, A., Pompella, G., Mazzola, A., Maccherini, M., Diciolla, F., Lazzerini, P.E., Abbracchio, M.P., Laghi-Pasini, F., 2006. Upregulation of A2A adenosine receptor expression by TNF-α in PBMC of patients with CHF: a regulatory mechanism of inflammation. J. Card. Fail. 11, 67–73.

Chan, K., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. Anal. Biochem. 157, 375–380.

Condie, R.M., Zak, S.J., Good, R.A., 1968. Effect of meningococcal endotoxin on the immune response. Proc. Soc. Exp. Biol. Med. 90, 355–360.

Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.F., Enjyoji, K., Linden, J., Oukka, M., Kuchroo, V.K., Strom, T.B., Robson, S.C., 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J. Exp. Med. 204, 1257–1265.

Di Virgilio, F., 2007. Purinergic signalling in the immune system. A brief update. Purinergic Signal. 3, 1–3.

Di Virgilio, F., Ceruti, S., Bramanti, P., Abbracchio, M.P., 2009. Purinergic signalling in inflammation of the central nervous system. Trends Neurosci. 32, 79–87.

Dombrowski, K.E., Ke, Y., Thompson, L.F., Kapp, J.A., 1995. Antigen recognition by CTL is dependent upon ectoATPase activity. J. Immunol. 154, 6227–6237.

Dombrowski, K.E., Ke, Y., Brewer, K.A., Kapp, J.A., 1998. Ecto-ATPase: an activation marker necessary for effector cell function. Immunol. Rev. 161, 111–118.

Dwyer, K.M., Deaglio, S., Gao, W., Friedman, D., Strom, T.B., Robson, S.C., 2007. CD39 and control of cellular immune responses. Purinergic Signal. 3, 171–180.

Erdmann, A.A., Gao, Z.G., Jung, U., Foley, J., Borenstein, T., Jacobson, K.A., Fowler, D.H., 2005. Activation of Th1 and Tc1 cell adenosine A2A receptors directly inhibits IL-2 secretion in vitro and IL-2-driven expansion in vivo. Blood 105, 4707–4714.

Ferrari, D., Pizzirani, C., Adinolfi, E., Lemoli, M.R., Curti, A., Idzko, M., Panther, E., Di Virgilio, F., 2006. The P2X7 receptor: a key player in IL-1 processing and release. J. Immunol. 176, 3877–3883.

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K.N., Linden, J., 2001. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol. Rev. 53, 527–552.

Genovese, T., Melani, A., Esposito, E., Mazzon, E., Di Paola, R., Bramanti, P., Pedata, F., Cuzzocrea, S., 2009. The selective adenosine A2A receptor agonist CGS 21680 reduces JNK MAPK activation in oligodendrocytes in injured spinal cord. Shock 32 (6), 578–585.

Goding, J.W., Grobben, B., Slegers, H., 2003. Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. Biochim. Biophys. Acta 1638, 1–19.

Hasko, G., Kuhel, D.G., Chen, J.F., Schwarzschild, M.A., Deitch, E.A., Mabley, J.G., Marton, A., Szabo, C., 2000. Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A2a receptor dependent and independent mechanisms. FASEB J. 14, 2065–2074.

Hasko, G., Cronstein, B.N., 2004. Adenosine: an endogenous regulator of innate immunity. Trends Immunol. 25, 33–39.

Hasko, G., Pacher, P., 2008. A2A receptors in inflammation and injury: lessons learned from transgenic animals. J. Leukoc. Biol. 83, 447–455.

Huang, S., Apasov, S., Koshiba, M., Sitkovsky, M., 1997. Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. Blood 90, 1600–1610.

Kreckler, L.M., Gizewski, E., Wan, T.C., Auchampach, J.A., 2009. Adenosine suppresses lipopolysaccharide-induced tumor necrosis factor-alpha production by murine macrophages through a protein kinase A- and exchange protein activated by cAMP-independent signaling pathway. J. Pharmacol. Exp. Ther. 331 (3), 1051–1061.

Koshiba, M., Rosin, D.L., Hayashi, N., Linden, J., Sitkovsky, M.V., 1999. Patterns of A2A extracellular adenosine receptor expression in different functional subsets of human peripheral T cells. Flow cytometry studies with anti-A2A receptor monoclonal antibodies. Mol. Pharmacol. 55, 614–624.

Lappas, C.M., Rieger, J.M., Linden, J., 2005. A2A adenosine receptor induction inhibits IFN-gamma production in murine CD4+ T cells. J. Immunol. 174, 1073–1080.

Lappas, C.M., Day, Y.J., Marshall, M.A., Engelhard, V.H., Linden, J., 2006. Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation. J. Exp. Med. 203, 2639–2648.

Linden, J., 2005. Adenosine in tissue protection and tissue regeneration. Mol. Pharmacol. 67, 1385–1387.

Martire, A., Calamandrei, G., Felici, F., Scattoni, M.L., Lastoria, G., Domenici, M.R., Tebano, M.T., Popoli, P., 2007. Opposite effects of the A2A receptor agonist CGS21680 in the striatum of Huntington's disease *versus* wild-type mice. Neurosci. Lett. 471, 78–83.

Medzhitov, R., 2008. Origin and physiological roles of inflammation. Nature 454, 428–435.

Nedeljkovic, N., Banjac, A., Horvat, A., Stojiljkovic, M., Nikezic, G., 2005. Developmental profile of NTPDase activity in synaptic plasma membranes isolated from rat cerebral cortex. Int. J. Dev. Neurosci. 23, 45–51.

Odashima, M., Bamias, G., Rivera-Nieves, J., Linden, J., Nast, C.C., Moskaluk, C.A., Marini, M., Sugawara, K., Kozaiwa, K., Otaka, M., Watanabe, S., Cominelli, F., 2005. Activation of A2A adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease. Gastroenterology 129, 26–33.

Ohta, A., Sitkovsky, M., 2001. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature 414, 916–920.

Ohta, A., Sitkovsky, M., 2009. The adenosinergic immunomodulatory drugs. Curr. Opin. Pharmacol. 9, 1–6.

Pawlinski, R., Pedersen, B., Kehrle, B., Aird, W.C., Frank, R.D., Guha, M., Mackman, N., 2003. Regulation of tissue factor and inflammatory mediators by Egr-1 in a mouse endotoxemia model. Blood 101, 3940–3947.

Pinhal-Enfield, G., Ramanathan, M., Hasko, G., Vogel, S.N., Salzman, A.L., Boons, G.J., Leibovich, S.J., 2003. An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A(2A) receptors. Am. J. Pathol. 163, 711–721.

Resta, R., Yamashita, Y., Thompson, L.F., 1998. Ecto-enzyme and signaling functions of lymphocyte CD73. Immunol. Rev. 161, 95–109.

Sakura, H., Nagashima, S., Nakahima, A., Maeda, M., 1998. Characterization of fetal serum 50-nucleotide phosphodiesterase: a novel function as a platelet aggregation inhibitor in fetal circulation. Thromb. Res. 91, 83–89.

Sevigny, C.P., Li, L., Awad, A.S., Huang, L., McDuffie, M., Linden, J., Lobo, P.I., Okusa, M.D., 2007. Activation of adenosine 2A receptors attenuates allograft rejection and alloantigen recognition. J. Immunol. 178, 4240–4249.

Sitkovsky, M.V., 2003. Use of the A(2A) adenosine receptor as a physiological immunosuppressor and to engineer inflammation in vivo. Biochem. Pharmacol. 65, 493–501.

Sitkovsky, M.V., Ohta, A., 2005. The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? Trends Immunol. 26, 299–304.

Sullivan, G.W., 2003. Adenosine A_{2A} receptor agonists as anti-inflammatory agents. Curr. Opin. Investig. Drugs 4, 1313–1319.

Sullivan, G.W., Fang, G., Linden, J., Scheld, W.M., 2004. Adenosine receptor activation improves survival in mouse models of endotoxemia and sepsis. J. Infect. Dis. 189, 1897–1904.

Thiel, M., Caldwell, C.C., Sitkovsky, M.V., 2003. The critical role of adenosine A_{2A} receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. Microbes Infect. 5, 515–526.

Vuaden, F.C., Cognato, G.P., Bonorino, C., Bogo, M.R., Sarkis, J.J.F., Bonan, C.D., 2007. Lipopolysaccharide alters nucleotidase activities from lymphocytes and serum of rats. Life Sci. 80, 1784–1791.

Vuaden, F.C., Fürstenau, C.R., Savio, L.E.B., Sarkis, J.J.F., Bonan, C.D., 2009. Endotoxemia alters nucleotide hydrolysis in platelets of rats. Platelets 20, 83–89.

Zimmermann, H., 2001. Ectonucleotidases: some recent developments and note on nomenclature. Drug Dev. Res. 52, 46–56.