UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

INFLUÊNCIA DA ENDOTOXEMIA E DA ATIVAÇÃO DOS RECEPTORES A2A DE ADENOSINA SOBRE A HIDRÓLISE DE NUCLEOTÍDEOS EXTRACELULARES

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Orientadora:

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Aos meus pais, que são a essência da minha vida

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Sumário

Resumo

O ATP extracelular atua como um mediador pró-inflamatório e a adenosina tem sido descrita por suas propriedades anti-inflamatórias. O papel desse nucleosídeo no controle da inflamação ocorre principalmente via receptores A2A. As ecto-enzimas responsáveis pelo controle dos níveis de nucleotídeos e nucleosídeos extracelulares são as ectonucleotidases. Esse grupo de ecto-enzimas é composto pela família das ecto-nucleosídeo trifosfato difosfoidrolases (E-NTPases), a família das ecto-nucleotídeo pirofosfatase/fosfodiesterases (E-NPP) e pela ecto-5'-nucleotidase. Considerando o papel que os nucleotídeos e nucleosídeos exercem durante eventos inflamatórios, e a importância das ectonucleotidases na manutenção dos seus níveis extracelulares, o objetivo deste trabalho foi investigar o efeito da indução do modelo de endotoxemia sobre as atividades ectonucleotidásicas em diferentes tipos e frações celulares. O envolvimento do receptor A2A sobre esses parâmetros também foi avaliado, através da utilização do agonista específico do receptor A2A, GCS-21680, no intuito de melhor compreender o envolvimento do sistema purinérgico no processo inflamatório. Para a indução do modelo de endotoxemia os ratos foram injetados intraperitonialmente (i.p.) com 2 mg/kg de LPS e os camundongos foram injetados i.p. com 12 mg/kg de LPS e/ou CGS-21680 (0,5 mg/kg, i.p.). As atividades ectonucleotidásicas foram determinadas em plaquetas de ratos, linfócitos de linfonodos mesentéricos de camundongos e preparações de membranas renais de camundongos. A análise da expressão das ectonucleotidases foi realizada através de RT-PCR. Os resultados demonstraram uma diminuição na hidrólise de ATP, ADP, AMP e 5'TMP em plaquetas de ratos após a indução da endotoxemia (28%, 28%, 30% e 26%, respectivamente). A contagem e a agregação plaquetária também foram diminuídas (40% e 55%, respectivamente). Em linfócitos de camundongos, houve um aumento na hidrólise de ATP, ADP, AMP e 5'TMP 24 horas após a injeção de LPS (178%, 111%, 207% e 62%, respectivamente) e 48 horas após a indução do modelo (135%, 178%, 121% e 116%, respectivamente). Quando o agonista do receptor A2A de adenosina, CGS-21680, foi co-administrado com o LPS, esse aumento foi revertido para as hidrólises de ATP, AMP e 5'-TMP. Em membranas renais de camundongos, os resultados demonstraram um aumento na hidrólise de ATP e 5'-TMP 48 horas após a injeção de LPS (48% e 47%, respectivamente) e a hidrólise do AMP foi diminuída 24 horas após a indução do modelo (40%). Os níveis extracelulares de ATP e adenosina foram diminuídos nos grupos tratados quando comparados ao controle em preparações de membranas renais. Os resultados deste trabalho indicam que as ectonucleotidases são moduladas pela endotoxemia em diferentes frações biológicas, sugerindo que as alterações observadas são conseqüência da resposta inflamatória. Em linfócitos esse efeito foi revertido pela ativação dos receptores A2A. Esses dados demonstram a interação cruzada entre a ativação dos receptores A2A de adenosina e as enzimas que modulam a produção do agonista desse receptor, bem como que a influência da ativação dos receptores A2A sobre as ectonucleotidases pode ser um dos mecanismos relacionados com as ações anti-inflamatórias e a resposta imune mediada por esse receptor.

Abstract

Extracellular ATP mainly functions as a proinflammatory mediator and adenosine has been reported to exert anti-inflammatory properties. A role for this nucleoside in the control of inflammation has been suggested acting mainly on adenosine A2A receptors. The ecto-enzymes responsible for the control of extracellular nucleotides and nucleosides levels are ectonucleotidases. This group of ectoenzymes is composed by the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the ecto-pyrophosphatase/phosphodiesterase (E-NPP) family and the ecto-5'-nucleotidase. 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 endotoxemia model on ectonucleotidase activities in different cells and cellular fractions. The involvement of A2A receptor under these parameters was also analyzed, utilizing CGS-21680, a specific A2A receptor agonist, in order to better understand the involvement of purinergic system in this process. For endotoxemia model induction, rats were injected intraperitoneally (i.p.) with 2 mg/kg LPS and mice were injected with 12 mg/kg and/or 0.5 mg/kg CGS-21680. Nucleotidase activities were determined in rat platelets, mice lymphocytes from mesenteric lymph nodes and mice kidney membrane preparations. Analysis of ectonucleotidase expression was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Results demonstrated a decrease on ATP, ADP, AMP and 5'TMP hydrolysis in rat platelets (28%, 28%, 30% and 26%, respectively), platelet account and aggregation (40% and 55%, respectively) after induction of endotoxemia model. In mice lymphocytes, we observed an increase in ATP, ADP, AMP and 5'TMP hydrolysis 24 hours after LPS injection (178%, 111%, 207% and 62%, respectively) and 48 hours after LPS injection (135%, 178%, 121% and 116%, respectively). When CGS-21680 was administered concomitant with LPS, this increase was reversed for ATP, AMP and 5'-TMP hydrolysis. In kidney membrane preparations of mice, results showed an increase on ATP and 5'-TMP hydrolysis 48 hours after LPS injection (48% and 47%, respectively) and 24 hours after LPS exposure there was a decrease on AMP hydrolysis (40%). The extracellular levels of ATP and adenosine were decrease in treated groups in kidney membrane preparations. These results indicate that endotoxemia modulates ectonucleotidases in different biological fractions, suggesting that these alterations are consequence of inflammatory response. The effect promoted by CGS-21680 in lymphocytes indicates a cross-talk between the A2A activation and the enzymes responsible for adenosine generation, but also that the influence of A2A receptor activation on ectonucleotidases might be one of the mechanisms related with its anti-inflammatory properties.

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Lista de Siglas

ADP – adenosina 5’-difosfato

AMP – adenosina 5’-monofosfato

Ap_nA - diadenosina polifosfato

ATP – adenosina 5’-trifosfato

CD39 – antígeno de ativação celular linfóide

CD73 – proteína de superfície de linfócitos

CGS-21680 - 3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(etilcarbomil)-3,4-dihidroxi-oxolan-2-il]purina-2-il]amino]etil]fenil]propanóico

CTI - centro de tratamento intensivo

CTP - citidina 5’-trifosfato

E-NTPDase – ecto-nucleosídeo trifosfato difosfoidrolase

E-NPP – ecto-nucleotídeo pirofosfatase/fosfodiesterase

IL - interleucina

IRA - injúria renal aguda

LPS – lipopolissacarídeo

NAD^+ - nicotinamida adenina dinucleotídeo

Pi – fosfato inorgânico

PKA - proteína cinase A

PKC - proteína cinase C

TLR – receptor do tipo “toll-like”

TNF – fator de necrose tumoral

TTP - timidina 5’ trifosfato

TXA2 – tromboxano A2

UDP – uridina 5’-difosfato

UTP - uridina 5’-trifosfato

5’-TMP - ρ-Nitrophenyl thymidine 5’-monophosphate

Introdução

1. Introdução

1.1. Processo Inflamatório

Inflamação é um mecanismo homeostático complexo que opera no sentido de promover uma resposta do organismo contra agentes nocivos endógenos e/ou exógenos; é uma resposta adaptativa desencadeada por um estímulo, como, por exemplo, uma infecção ou dano tecidual (Barton, 2008; Medzhitov, 2008). O enciclopedista romano *Aulus Cornelius Celsus* (25 a.C - 50 d.C.) foi o primeiro a listar os quatro sinais cardinais da inflamação: *calor* (aumento da temperatura corporal no local), *rubor* (hiperemia), *tumor* (edema, inchaço) e *dolor* (dor). Esses sintomas descrevem e sumarizam os eventos cruciais que ocorrem no tecido durante o processo inflamatório (Di Virgilio, 2009). Quando um estímulo inflamatório é gerado no hospedeiro, ocorre um desequilíbrio homeostático e o organismo tenta restaurar esse balanço. Se esse mecanismo não for efetivo, o quadro inflamatório pode se agravar, chegando, em casos mais graves, ao desenvolvimento da sepse (Medzhitov, 2008).

A sepse é definida como uma síndrome de resposta inflamatória sistêmica (SIRS) que ocorre como conseqüência de uma infecção (Bone et al., 1992) e pode ser considerada o resultado da produção de um largo espectro de mediadores inflamatórios, atuando em resposta a ecto- e endotoxinas bacterianas presentes na circulação (Thiel et al., 2003). Apesar dos avanços com relação ao cuidado dos pacientes e da introdução de potentes agentes antimicrobianos, a mortalidade permanece em torno de 20%, sendo a principal causa de mortes em centros de terapia intensiva (CTI) (Martin et al., 2003).

Fatores de origem microbiana, como as endotoxinas, são liberados por bactérias e agem na superfície de células hospedeiras, onde se ligam aos receptores e induzem a liberação de citocinas, produzindo sintomas locais ou sistêmicos. Dentre os mediadores

inflamatórios liberados pelo hospedeiro, destacam-se as quimiocinas (como a interleucina IL-8), as citocinas pró-inflamatórias (como o fator de necrose tumoral [TNF]-α e as interleucinas IL-1β, IL-6 e IL-12), as citocinas anti-inflamatórias (IL-10, por exemplo) (Van der Poll e Van Deventer, 1999), eicosanóides (lipídeos biologicamente ativos, como prostaglandinas e leucotrienos) (O'Donnel et al., 2009) e óxido nítrico (Symeonides e Balk, 1999; Víctor et al., 2009).

1.1.2. O papel do lipopolissacarídeo (LPS)

O lipopolissacarídeo é um componente da membrana externa das bactérias Gram-negativas e seu papel como adjuvante na resposta imune adaptativa é conhecido há muito tempo (Condie et al., 1968). As bactérias Gram-negativas apresentam um envoltório celular bilateralizado e assimetricamente organizado. O LPS é o constituinte predominante da membrana externa, correspondendo a cerca de três quartos da superfície celular de *Escherichia coli*, sendo o restante composto por proteínas (Nikaido e Vaara, 1987). A integridade da membrana celular, e do LPS que a constitui, é essencial para a viabilidade bacteriana. Um exemplo disso é o fato de que mutantes incapazes de produzir LPS não são viáveis (Rietschel et al., 1994). Devido ao seu papel crucial e a sua localização, o LPS pode ser um alvo ideal para o ataque de anticorpos e agentes imunológicos ou farmacológicos (Ianaro et al., 2009).

O LPS representa um imunomodulador altamente ativo, capaz de induzir resistência não específica a infecções virais e bacterianas e atua como um dos principais fatores responsáveis por manifestações tóxicas de infecções Gram-negativas severas e inflamação generalizada (Rietschel e Brade, 1992; Bone, 1993; Leon et al., 2008). Programas de pesquisa no campo das doenças infecciosas objetivam a neutralização ou

a eliminação dessa endotoxina da circulação através do desenvolvimento de vacinas específicas (Cross et al., 2004).

Como observado na Figura 1, os lipopolissacarídeos bacterianos são compostos de um domínio hidrofóbico, denominado lipídeo A (endotoxina), uma parte central (correspondente ao núcleo), e um polissacarídeo distal (antígeno-O):

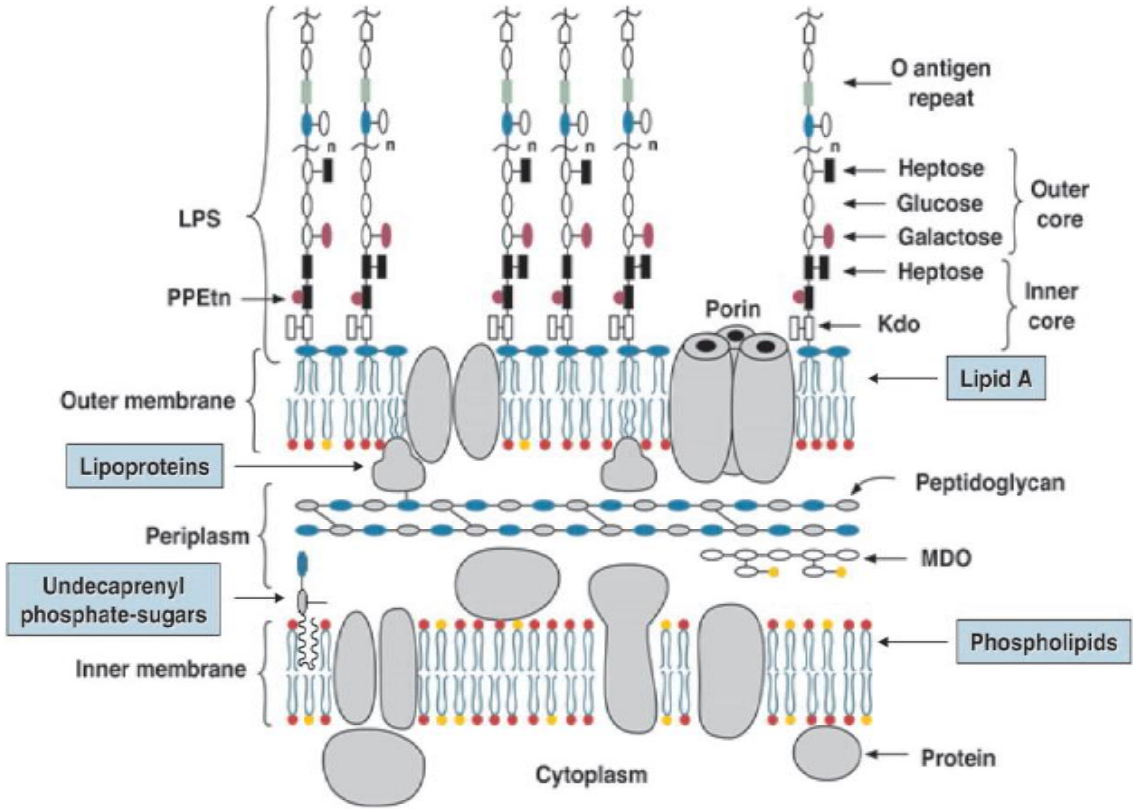

Figura 1: Modelo de membranas interna e externa de *Escherichia coli* K-12. O LPS é um componente essencial da membrana externa de bactérias Gram-negativas. Corresponde a um complexo glicolipídico composto de um polissacarídeo hidrofílico e um domínio hidrofóbico, conhecido como lipídeo A, o qual é o responsável pela atividade biológica do LPS. Extraído de Raetz e Whitfield (2002).

Os receptores responsáveis pelo reconhecimento de patógenos e subsequente indução da resposta inflamatória são os receptores do tipo “toll-like” (TLR) (Underhill,

2004; Gay e Gangloff, 2007). Ligantes de TLR são conhecidos por atuarem como adjuvantes, intensificando a resposta do sistema imune adaptativo (Hoebe et al., 2004). Nessa família de receptores transmembrana, o receptor específico responsável pelo reconhecimento do LPS é o TLR4 (Akira et al., 2001; Beutler e Rietschel, 2003). Na Figura 2, pode-se observar como é iniciada a resposta inflamatória através da estimulação desse receptor pelo LPS.

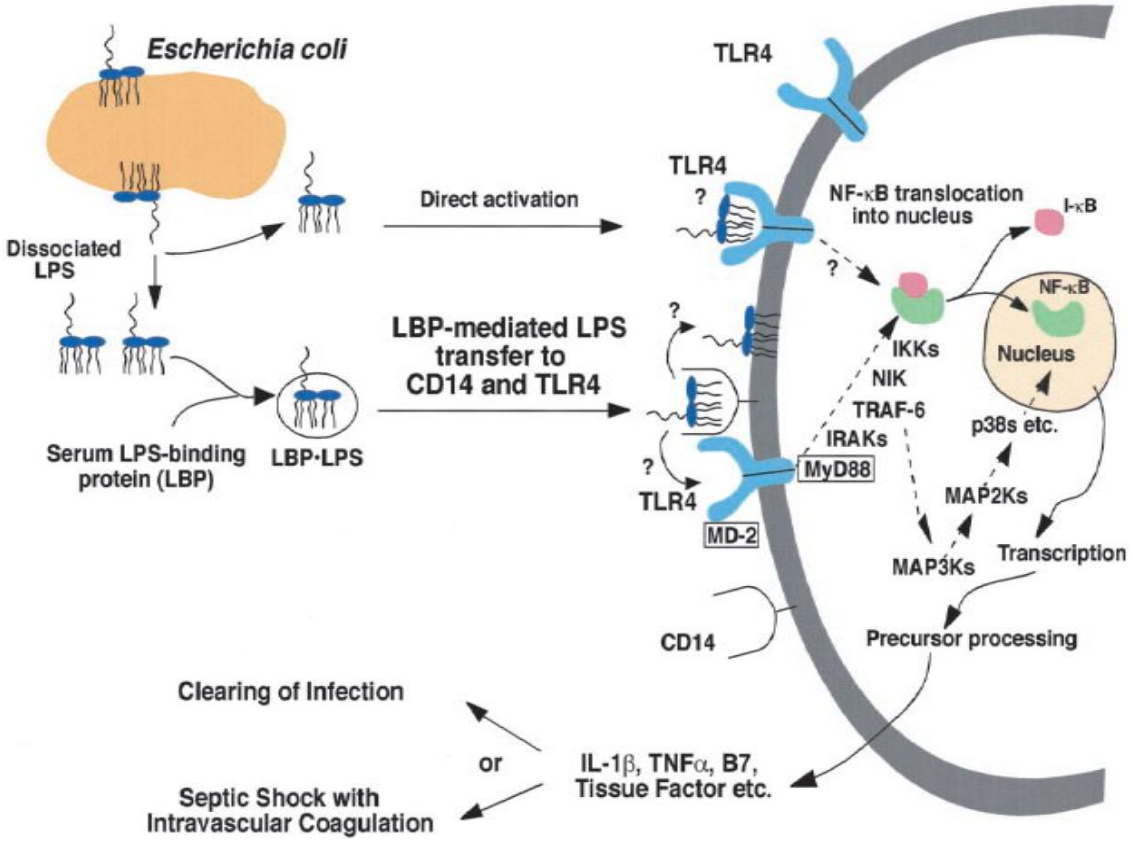

Figura 2: Detecção do lipídeo A pelo receptor do sistema imune de células animais TLR4. Em situações de lise ou multiplicação bacteriana, pode ocorrer a liberação sistêmica do LPS, e o subsequente reconhecimento do lipídeo A pelo TLR4 representa o primeiro passo na sinalização desse receptor. A interação entre o lipídeo A e o TLR4 envolve moléculas acessórias, incluindo a glicoproteína de superfície de membrana CD14, bem como a proteína solúvel acessória MD2. A informação é transmitida via

uma cascata de sinalização molecular até o núcleo da célula, para que uma adequada resposta celular, tal como a indução de expressão de citocinas, por exemplo, possa ser iniciada. A estimulação de receptores TLR4 pelo LPS pode iniciar uma resposta imune pró-inflamatória, a qual serve para eliminar a infecção bacteriana, mas se a resposta não for suficiente pode resultar em dano tecidual ao hospedeiro, culminando em choque endotóxico. Extraído de Raetz e Whitfield (2002).

1.1.3. Agregação Plaquetária Durante Inflamação

As plaquetas são fragmentos celulares anucleados liberados na circulação sanguínea através da fragmentação de megacariócitos (Hartwig e Italiano, 2003). Sob condições normais, as plaquetas circulam livremente no sangue e não se aderem umas às outras ou às paredes dos vasos. Sua principal ação é assegurar a homeostase primária, o que significa a manutenção da integridade dos vasos sanguíneos e a rápida interrupção do sangramento nos eventos de injúria vascular (Jurk e Kehrel, 2005). A função plaquetária pode ser vista como uma sucessão de eventos sobrepostos, envolvendo adesão, agregação, secreção e promoção da atividade pró-coagulante (Bennett et al., 1990; Bithell, 1993).

Além de estancar a perda de sangue e reparar a injúria vascular, é crescente a aceitação de que as plaquetas são células inflamatórias com outros papéis funcionais (Weyrich et al., 2003; McNicol et al., 2008) e que a agregação plaquetária também pode ocorrer como conseqüência de um evento inflamatório (Piro et al., 2005). A inflamação sistêmica é um potente estímulo pró-trombótico; durante a inflamação crônica e aguda, as plaquetas podem desempenhar importantes funções, incluindo a liberação de mediadores pró-inflamatórios, exposição de moléculas de superfície que apresentam

ação inflamatória e interação com leucócitos e células endoteliais (Elstad et al., 1995; McIntyre et al., 2003).

Após a sua ativação, as plaquetas liberam compostos ativos, os quais incluem fatores de crescimento, como o fator de crescimento derivado de plaquetas (PDGF) e citocinas pró-inflamatórias (Gawaz et al., 2000); expressam P-seletina (André, 2004) e CD40L (ligante de CD40 ou CD154, um membro da família TNF) (Henn et al., 1998). Esses mediadores inflamatórios promovem a expressão de moléculas de adesão nas células endoteliais e o recrutamento e extravasamento de monócitos (Henn et al., 1998; Lindemann et al., 2001; Gawaz et al., 1998), contribuindo para as respostas inflamatórias e pró-coagulantes (Huo e Ley, 2004).

A relação entre a agregação plaquetária e os eventos inflamatórios é controversa. Em modelos animais, alguns estudos demonstraram a acentuada agregação plaquetária induzida por endotoxina (Alqmvist et al., 1983; Beijer et al., 1987; Matera et al., 1992; Itoh et al., 1996). Outros trabalhos demostraram que, *in vitro*, compostos bacterianos como o LPS e o ácido lipoprotéico de *Staphylococcus aureus* podem se ligar a membranas de plaquetas e células endoteliais de pacientes sépticos e inibir a agregação plaquetária (Salden e Bas, 1994; Sheu et al., 2000; Leytin et al., 2002). Entretanto, citocinas geradas durante o evento séptico não foram observadas ativando plaquetas de humanos tanto diretamente quanto via trombina (Leytin et al., 2002).

Em estudos clínicos também se observa diferenças entre os resultados apresentados. Gawaz e colaboradores (1997) reportaram que a agregação plaquetária aumentava durante o evento séptico, enquanto diversos pesquisadores demostraram uma diminuição da agregação plaquetária (Cowan et al., 1976; Boldt et al., 1997; Yaguchi et al., 2004).

1.1.4. Injúria Renal Aguda

Injúria Renal Aguda (IRA) é uma condição que afeta cerca de 8% dos pacientes hospitalares e mais de 50% dos pacientes internados em CTI (Uchino et al., 2006; Hoste et al., 2006), sendo, assim, um dos maiores problemas em hospitais e CTI. Existem fortes evidências de que a sepse severa e o choque séptico são as maiores causas de IRA em pacientes internados no CTI e explicam mais de 50% dos casos de IRA nessas unidades (Silvester et al., 2001; Uchino et al., 2005). Apesar dos avanços nos cuidados aos pacientes, a mortalidade permanece alta (Silvester et al., 2001; Uchino et al., 2005; Bagshaw et al., 2007).

Mesmo sendo muito importante a compreensão da patogênese da IRA, muito ainda precisa ser estudado. Como demonstrado na revisão de Bellomo e colaboradores, publicada em 2008, os mecanismos da IRA ainda são poucos esclarecedores, mas alguns pontos já podem ser elucidados. Inicialmente, a IRA séptica foi considerada um efeito secundário, resultante da isquemia renal, mas um crescente número de dados experimentais tem refutado essa ideia. Alguns estudos têm demonstrado que a circulação renal participa da vasodilatação sistêmica observada durante eventos de choque séptico e/ou sepse severa, e que o fluxo sanguíneo renal não é diminuído, sendo que o desenvolvimento da IRA séptica não ocorre em decorrência de hipoperfusão renal, mas sim de adequada, e em alguns casos elevada, perfusão renal (Ravikant e Lucas, 1977; Brenner et al., 1990; Langenberg et al., 2005; Langenberg et al., 2007). A Figura 3 demonstra um dos possíveis mecanismos que envolvem a diminuição da taxa de filtração glomerular durante um evento séptico, apesar do aumento do fluxo sanguíneo renal.

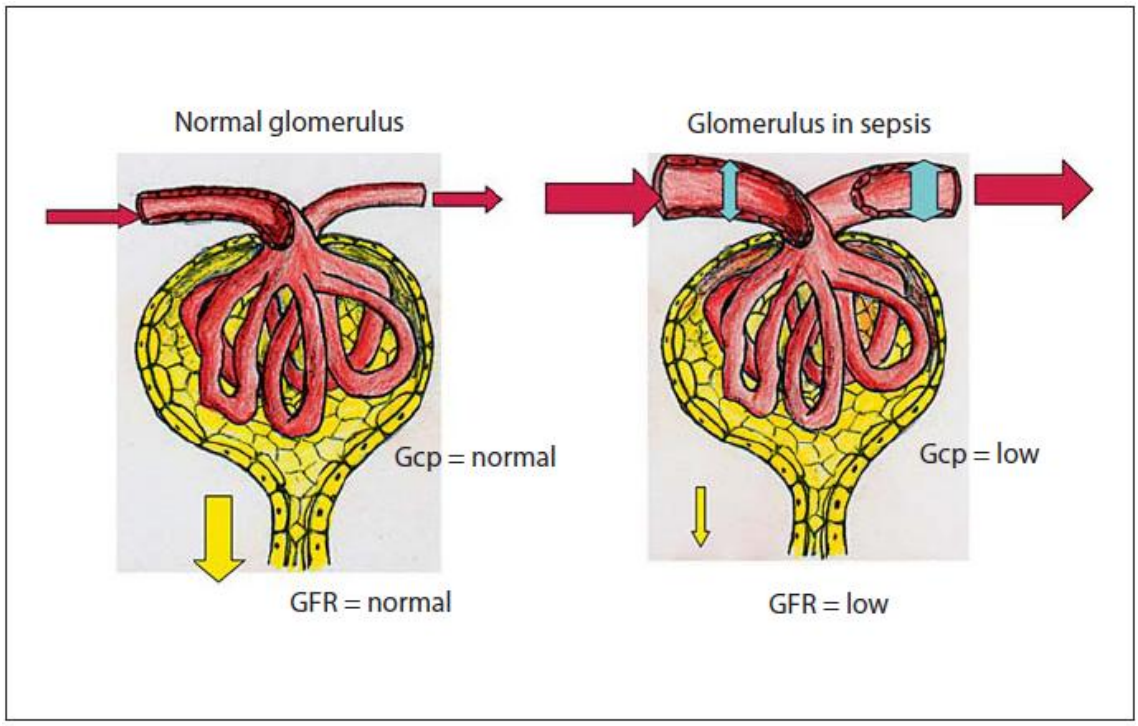

Figura 3: Possível mecanismo para explicação da diminuição da taxa de filtração glomerular durante um evento séptico. O glomérulo durante evento um séptico apresenta vasodilatação arteriolar aferente e eferente, mas a vasodilatação eferente é maior, como demonstrado pela seta azul mais larga. Isso explica o fato de que, mesmo com o fluxo sanguíneo renal aumentado, a pressão capilar glomerular, a taxa de filtração glomerular e a produção de urina se apresentam diminuídas. Extraído de Bellomo e colaboradores, 2008.

1.2. Sinalização Purinérgica e Inflamação

Muitos metabólitos e as enzimas responsáveis pela sua geração são considerados verdadeiros mediadores inflamatórios, como, por exemplo, óxido nítrico/óxido nítrico sintase, arginina/arginase, lipoxina/lipoxigenase e, mais recentemente, nucleotídeos e o nucleosídeo da adenina, bem como as enzimas responsáveis pelo seu metabolismo (Di Virgilio et al., 2009). Os nucleotídeos e nucleosídeos extracelulares são importantes

moléculas sinalizadoras, sendo essenciais para o início e a manutenção de reações inflamatórias. Entre suas ações, estão o recrutamento de leucócitos e de mastócitos ao sítio inflamatório, a ativação da vasculatura e o prolongamento da ativação inflamatória (Luttikhuizen et al., 2004).

1.2.1. Receptores Purinérgicos

Os nucleotídeos da adenina e o nucleosídeo adenosina podem exercer seus efeitos através da ativação de receptores purinérgicos que são divididos em dois grandes grupos: receptores do tipo P1 e receptores do tipo P2. Os purinoceptores do tipo P1 são ativados por adenosina, enquanto os purinoceptores do tipo P2 são mais eficientemente ativados por ATP (Burnstock, 2009).

Os receptores purinérgicos do tipo P2 são subdivididos em duas subclasses: P2X e P2Y. A família P2X está acoplada a canais iônicos e está envolvida na transmissão excitatória rápida, enquanto a família P2Y é composta por receptores metabotrópicos acoplados à proteína G. Dentre os subtipos de receptores, sete da família P2X (P2X1-7) e oito de receptores P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14) são farmacologicamente distintos e possuem suas respostas funcionais descritas (Gever et al., 2006; Burnstock, 2007).

Os purinoceptores do tipo P1 possuem quatro subtipos: A1, A2A, A2B e A3 (Fredholm et al., 2001; Burnstock 2009). Os receptores de adenosina transmitem seu sinal principalmente via ativação de proteínas G heterotriméricas, que podem tanto estimular (Gs) quanto inibir (Gi) a atividade da adenilato ciclase, a enzima que catalisa a formação de AMP cíclico. Os receptores do tipo A1 e A3 inibem a adenilato ciclase e são receptores de alta e baixa afinidade para adenosina, respectivamente. Em contrapartida, os receptores A2A e A2B, de alta e baixa afinidade, respectivamente,

ativam a adenilato ciclase. Além de regular a atividade da adenilato ciclase, os subtipos de receptores de adenosina são também acoplados a distintas proteínas G, atuando em outros sistemas efetores, os quais incluem canais de cálcio e potássio, fosfolipase C, -D, -A2, GMP cíclico, fosfodiesterases e proteínas quinases ativadas por mitógenos, atuando na modulação de diferentes funções celulares (Fredholm et al., 2000; Burstock, 2007).

1.2.2. ATP

Sob condições fisiológicas, o ATP é co-liberado com uma grande quantidade de neurotransmissores, os quais incluem acetilcolina, norepinefrina, glutamato, ácido γ-aminobutírico e neuropeptídeo Y (Burnstock, 2004a). O ATP tem sua liberação marcadamente aumentada em situações de hipóxia, insulto isquêmico, inflamação ou trauma. Indícios do envolvimento do ATP extracelular na inflamação sistêmica datam da década de 1970, quando Dahlquist e Diamant reportaram que o ATP estava envolvido na liberação de histamina de mastócitos de ratos. No ano de 1980, Cockcroft e Gomperts demonstraram a expressão de receptores específicos para ATP, posteriormente identificados como o subtipo P2X7, igualmente em mastócitos. Durante o processo inflamatório, o ATP exerce uma série de efeitos. Está envolvido no desenvolvimento da inflamação por uma combinação de ações: liberação de histaminas de mastócitos, provocando produção de prostaglandinas e produção e liberação de citocinas de células do sistema imune (Di Virgilio et al., 1998). Esse nucleotídeo induz a produção de resposta em linfócitos, monócitos e granulócitos polimorfonucleares (Di Virgilio et al., 2001).

A participação do ATP no processo inflamatório ganhou maior aceitação após os receptores do tipo P2 serem clonados e sua expressão pelas células inflamatórias ter

sido completamente caracterizada. O ponto crucial foi a descoberta da forte ligação do receptor P2X7 na maturação e secreção de interleucina (IL)-1 (Perregaux e Gabel, 1994; Ferrari et al., 1997; Di Virgilio, 2007) e a demonstração *in vivo* de que o ATP está presente em altos níveis no espaço extracelular durante a inflamação (Idzko et al., 2007; Pellegati et al., 2008). Entre os outros receptores P2 tem sido revelada uma estreita ligação com o processo inflamatório. Pode-se citar o papel exercido pelo receptor P2Y2 na orientação de neutrófilos em um gradiente quimiotático (Chen et al., 2006), a implicação do receptor P2Y6 na inflamação intestinal e na liberação de IL-6 e IL-8 (Gribc et al., 2008), bem como o papel do receptor P2Y11 na maturação diferenciada de células dendríticas humanas (Ia Sala et al., 2001; Schnurr et al., 2003).

1.2.3. ADP

Os nucleotídeos extracelulares e seus receptores exercem importantes papéis no sistema cardiovascular, incluindo ativação plaquetária; vasodilatação e vasoconstrição, dependendo da presença ou ausência de endotélio, respectivamente; e controle do tônus vascular pelos nervos perivasculares (Burnstock, 2002). Inicialmente, o ADP foi identificado como um fator derivado de eritrócitos que influenciava a adesão plaquetária ao vidro (Gaarder et al., 1961) e induzia agregação plaquetária (Born, 1962). Nas plaquetas, o ADP liga-se a receptores metabotrópicos do tipo P2Y1 e P2Y12. O receptor P2Y1 tem um papel crucial no início da ativação plaquetária induzida por ADP e colágeno. Por outro lado, o receptor P2Y12 tem sido mais bem conhecido e caracterizado em suas evidências genéticas e farmacológicas. Esse receptor está envolvido na potenciação da secreção plaquetária (Cattaneo et al., 2000) e exerce um papel central na amplificação da agregação induzida por todos os conhecidos agonistas

de plaquetas, os quais incluem colágeno, trombina, complexos imunes, tromboxano A2, adrenalina e serotonina (Conley e Delaney, 2003; Hechler et al., 2005).

1.2.4. Adenosina

A adenosina é uma molécula sinalizadora endógena que regula numerosos processos fisiológicos e patológicos (Fredholm et al., 2001) A adenosina é produzida em resposta a situações de estresse metabólico ou dano celular e elevações nas concentrações de adenosina extracelular ocorrem em situações de isquemia, hipóxia, inflamação e trauma (Haskó e Cronstein, 2004). Em situações homeostáticas, os níveis de adenosina extracelular permanecem em concentrações nanomolares (10 a 200 nM), já em situações de hipóxia ou estresse tecidual os níveis de adenosina são elevados a concentrações micromolares (10 a 100 μM) (Fredholm, 2007).

A fonte mais importante para aumento nos níveis de adenosina extracelular durante situações de estresse metabólico é a liberação do ATP intracelular, seguido do catabolismo extracelular desse nucleotídeo, até a geração da adenosina, por ação das ectonucleotidases. O controle da sinalização celular exercida pela adenosina é realizado pelo seu catabolismo a inosina, através da ação da adenosina deaminase, ou pela sua recaptação e refosforilação a ATP, via adenosina quinase (Haskó e Pacher, 2008). Evidências demonstram que a adenosina é uma importante molécula sinalizadora e que os receptores desse nucleosídeo são importantes alvos moleculares na patofisiologia da inflamação. Todas as células inflamatórias expressam receptores de adenosina e diversos estudos têm sido realizados no intuito de desenvolver intervenções terapêuticas para o controle da inflamação via receptores de adenosina (Jacobson et al., 2006; Fernandez et al., 2008; Blackburn et al., 2009; Ohta e Sitkovsky, 2009).

Dentre os quatro subtipos de receptores de adenosina, diversos estudos têm demonstrado a participação da vias de sinalização do receptor A2A na limitação da inflamação e dano tecidual (Haskó e Cronstein, 2004; Linden, 2005; Sitkovsky e Ohta, 2005; Haskó e Pacher, 2008). Um dos primeiros estudos demonstrando as propriedades anti-inflamatórias desse receptor foi realizado em 1983, por Cronstein e colaboradores, que demonstraram que, através da ativação do receptor A2A, ocorre uma diminuição na formação de superóxido em neutrófilos. A importância do receptor A2A na modulação da inflamação é observada através das suas propriedades anti-inflamatórias, as quais incluem a inibição da ativação de células T (Huang et al., 1997; Erdmann et al., 2005) e o controle na produção de mediadores inflamatórios como IL-12, TNF-α e INFγ por monócitos (Haskó et al., 2000), células dendríticas (Panther et al., 2003) e células T (Lappas et al. 2005).

Tem sido bastante investigado o uso de agonistas de receptores A2A no tratamento de eventos inflamatórios e sepse (Thiel et al., 2003; Sullivan et al., 2004). Dados genéticos e farmacológicos indicam que a ativação de receptores A2A inibe a inflamação induzida por LPS, atuando em diferentes tipos celulares (Sullivan et al., 1999; Hogan et al., 2001). Agonistas deste receptor diminuem a concentração sérica de TNF-α em camundongos com endotoxemia (Haskó et al., 1996) e inibem a liberação de IL-12 e TNF-α induzida por LPS em macrófagos isolados de camundongos (Haskó et al., 2000). Agonistas A2A diminuem a liberação de produtos oxidativos e não oxidativos de neutrófilos ativados (Cronstein et al., 1983; Richter, 1992; Bouma et al., 1997; Sullivan et al., 2001) e diminuem a adesão dos neutrófilos a superfícies biológicas (Cronstein et al., 1992; Okusa et al., 2001).

Patógenos liberam fatores que ativam a imunidade inata tanto diretamente (através do LPS, por exemplo) quanto indiretamente (através da ruptura de células do

hospedeiro e liberação de ATP, por exemplo). No espaço extracelular o ATP é degradado pela ação das ectonucleotidases e a adenosina é gerada, exercendo seus efeitos anti-inflamatórios, principalmente via ativação do subtipo A2A. A figura 4 demonstra os efeitos promovidos pelo ATP e adenosina durante a inflamação.

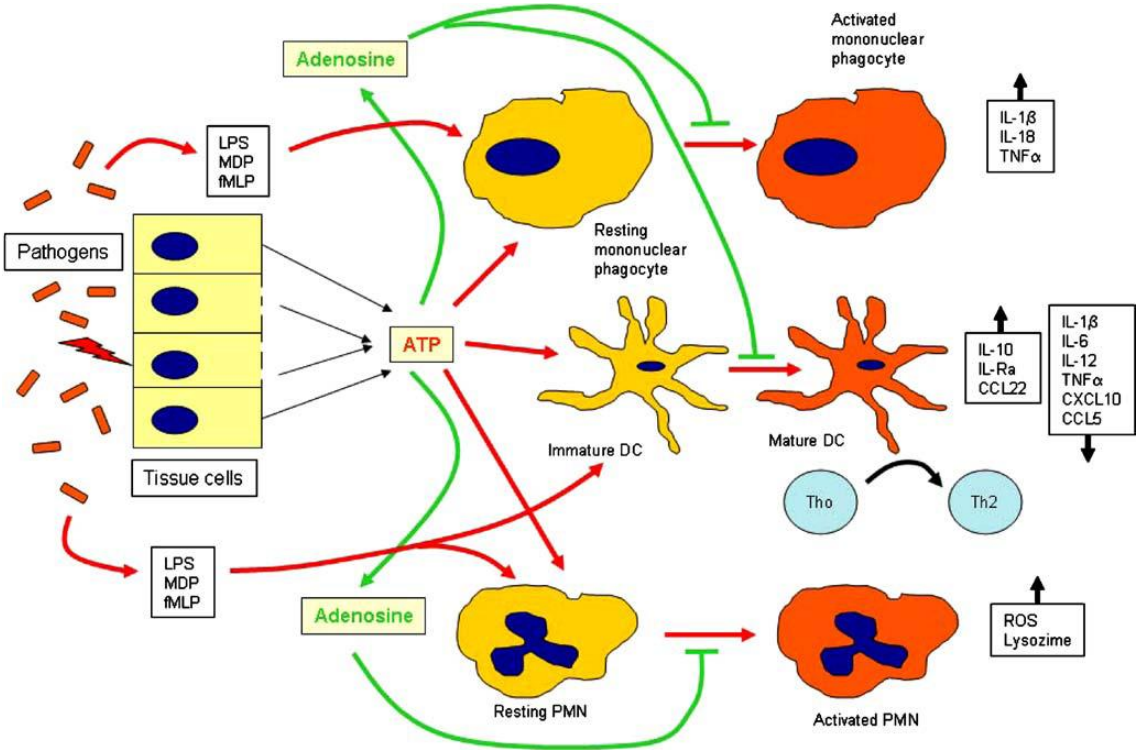

Figura 4: Sinalização purinérgica na ativação/desativação da resposta imune inata. Fatores bacterianos recrutam e estimulam macrófagos teciduais, células dendríticas (DC) e leucócitos polimorfonucleares (PMN). As células inflamatórias ativadas são demonstradas em vermelho. O ATP extracelular, que é acumulado nos sítios de inflamação e pode ser considerado como um sinal endógeno de perigo, modula a atividade dos fatores derivados de patógenos de diferentes maneiras (destacado pelas setas vermelhas). Por exemplo, o ATP pode exercer um efeito proinflamatório sinergístico, através do aumento da liberação de citocinas (IL-1b, IL-8 e TNFα). A adenosina gerada pela degradação do ATP extracelular exerce seus efeitos anti-inflamatórios via ativação dos receptores P1 (destacado pelas setas verdes). Extraído de Di Virgílio, 2007.

1.2.5. Ectonucleotidases

Os nucleotídeos presentes no meio extracelular podem ser hidrolisados por uma variedade de enzimas que estão localizadas nas membranas celulares ou presentes no meio intracelular e/ou extracelular na forma solúvel (Zimmermann, 2001). Essas enzimas são denominadas ectonucleotidases e exercem um papel crucial no controle da homeostasia dos níveis de nucleotídeos e nucleosídeos extracelulares. As enzimas que estão localizadas nas membranas celulares estão ancoradas na membrana plasmática e possuem seu sítio catalítico voltado para o meio extracelular. Dentre elas, pode-se destacar a família das ecto-nucleosídeo trifosfato difosfoidrolases (E-NTPases), que são responsáveis pela hidrólise de nucleotídeos trifosfatados e difosfatados até os seus respectivos nucleotídeos monofosfatados. A família da ecto-5´- nucleotidase, também pertencente a este grupo de ecto-enzimas, é responsável pela hidrólise de nucleotídeos monofosfatados aos seus respectivos nucleosídeos. Outra família de ecto-enzimas que estão envolvidas na hidrólise de nucleotídeos extracelulares é a da ecto-nucleotídeo pirofosfatase/fosfodiesterase (E-NPP), que é capaz de hidrolisar 3',5'-cAMP, ATP, ADP, NAD^+ e Ap_nA (Zimmermann, 1999; Zimmermann, 2001; Yegutkin, 2008).

1.2.5.1. E-NTPDases

Todos os membros de superfície celular da família das NTPDases são proteínas glicosiladas com massa molecular de cerca de 70 a 80 kDa e que apresentam grande reatividade imunológica (Zimmermann, 2000). Estas enzimas estão firmemente ligadas à membrana plasmática via dois domínios transmembrana com regiões N- e C-terminais e o seu sítio ativo está voltado para o meio extracelular. Apresentam também cinco regiões altamente conservadas na porção central hidrofóbica, que são conhecidas como

"regiões conservadas da apirase" (Robson et al., 2006). Com relação à hidrólise de nucleotídeos, a NTPDase1 (conhecida como ecto-apirase, ecto-ATP difosfoidrolase e CD39, sendo inicialmente reconhecida como um antígeno de ativação celular linfóide) hidrolisa ATP e ADP igualmente bem, sendo a proporção da hidrólise destes dois substratos de 1:1 (Heine et al., 1999; Zimmermann, 2001). A NTPDase2 (também nomeada CD39L1, ecto-ATPase) hidrolisa o ATP 30 vezes mais que o ADP (Kirley, 1997; Zimmermann, 2001). A NTPDase3 (conhecida também por CD39L3, HB6) e a NTPDase8 preferem o ATP em relação ao ADP numa razão de hidrólise de aproximadamente 3:1 e 2:1, respectivamente. (Zimmermann, 2001; Lavoie et al., 2004; Bigonnesse et al., 2004).

As outras enzimas representantes dessa família são a NTPDase4, 5, 6 e 7. A NTPDase4 tem sido localizada no aparelho de Golgi (UDPase, NTPDase4β) e em vacúolos lisossômicos/autofágicos (NTPDase4α). A NTPDase4α apresenta uma alta preferência por UTP e TTP, enquanto a NTPDase4β por CTP e UDP e a função destas enzimas ainda não está bem esclarecida (Zimmermann, 2001). A NTPDase5 (CD39L4, ER-UDPase) e a NTPDase6 (CD39L2) que são ativadas por cátions divalentes, têm uma preferência maior por nucleotídeos difosfatados. Acredita-se que a NTPDase5 e a NTPDase6 participam das reações de glicosilação envolvidas nos processos de dobramento de glicoproteínas (Zimmermann, 2001). Já a NTPDase7 (LALP1) prefere nucleosídeos trifosfatados como substrato e está localizada em vesículas intracelulares (Shi et al., 2001).

1.2.5.2. Ecto-5'-nucleotidase

Segundo Yegutkin (2008), até o momento, sete membros da família das 5'-nucleotidases já foram isolados e caracterizados, sendo que cinco encontram-se localizados no citosol, um na matriz mitocondrial e um ancorado externamente à membrana plasmática. A ecto-5'-nucleotidase consiste de duas subunidades glicoprotéicas, com massas moleculares de cerca de 60 a 70 kDa e estão ancoradas à membrana plasmática por uma molécula de glicosil fosfatidil inositol (GPI) (Zimmermann, 1996). Essa ecto-enzima é também conhecida como CD73 e representa um marcador de maturação de linfócitos B e T (Hunsucker et al., 2006). A ecto-5'-nucleotidase pode revelar uma variedade de funções, dependendo de sua expressão celular e tecidual. Essa enzima pode estar envolvida na adesão celular e exerce um importante papel na formação de adenosina a partir da hidrólise do AMP extracelular e subseqüente ativação dos receptores P1 de adenosina (Zimmermann, 2001).

1.2.5.3. E-NPP

As enzimas da família E-NPP possuem ampla distribuição nos tecidos e sete membros são conhecidos (NPP1-7). Elas são capazes de hidrolisar 3’5’-cAMP a AMP e Pi, ADP a AMP e Pi, NAD^+ a AMP e nicotinamida mononucleotídeo, e diadenosina polifosfato (Ap_nA) a Ap_{n-1} e AMP, sendo que nucleotídeos púricos e pirimidínicos servem como substrato. Além disso, podem hidrolisar ligações fosfodiéster de ácidos nucléicos e ligações pirofosfato de nucleotídeos (Stefan et al., 2005).

Apenas os três primeiros membros dessa família, a NPP1 (inicialmente conhecida como antígeno de diferenciação plasmática celular-1), a NPP2 (também denominada autotaxina, fosfodiesterase 1α) e a NPP3 (fosfodiesterase 1β) são capazes

de hidrolisar vários nucleotídeos sendo, consequentemente, relevantes no contexto da sinalização purinérgica (Goding et al., 2003). As NPP1-3 são metaloenzimas, com massa molecular de cerca de 110 a 125 kDa, caracterizadas por uma estrutura modular similar composta de um curto domínio intracelular (N-terminal intracelular), um domínio transmembrana simples e um domínio extracelular contendo um sítio catalítico conservado (Goding et al., 2003).

1.2.5.4. Ectonucleotidases e Inflamação

Alguns estudos apresentam fortes indícios sobre o papel das ectonucleotidases como mediadoras do processo inflamatório. Trabalhos demonstram que a NTPDase1 (CD39) tem sua atividade e expressão aumentadas em plaquetas de pacientes com hipercolesterolemia (Duarte et al., 2007) e em linfócitos de pacientes com esclerose múltipla (Spanevello et al., 2010). Em linfócitos de linfonodos mesentéricos de ratos submetidos ao modelo de endotoxemia foi observado um aumento na hidrólise de nucleotídeos tri-, di- e monofosfatados, enquanto em soro a hidrólise de ATP, ADP, AMP e 5'-TMP foi diminuída (Vuaden et al., 2007). Camundongos *knockout* para CD73 apresentam maior migração de linfócitos aos linfonodos 24 horas após injeção de LPS (Takedachi et al., 2008) e a expressão dessa enzima é aumentada durante inflamação induzida por colite (Louis et al., 2008). Reutershan e colaboradores (2009) demonstraram a expressão aumentada de CD39 e CD73 após a indução de injúria pulmonar via administração de LPS, revelando a mediação na migração de neutrófilos pulmonares exercida por essas enzimas.

1.3. Objetivos

Objetivo Geral

Muitos estudos descrevem a importância dos nucleotídeos no desenvolvimento do processo inflamatório e o papel da adenosina na supressão de tal processo. Entretanto, poucos estudos discutem o papel exercido pelas ectonucleotidases. Estas ecto-enzimas são responsáveis pela hidrólise do ATP e o subsequente término da sua resposta inflamatória, bem como pelo aumento nas concentrações extracelulares de adenosina, desencadeando suas respostas anti-inflamatórias. Sendo assim, este estudo objetiva analisar as atividades ectonucleotidásicas em diferentes frações e tipos celulares após a indução de endotoxemia em modelos animais. O envolvimento do receptor A2A de adenosina sobre esses parâmetros também foi investigado, no intuito de obter maiores esclarecimentos sobre o papel exercido pelo sistema purinérgico durante o processo inflamatório.

Objetivos Específicos

I. Verificar o efeito *in vitro* de diferentes concentrações de LPS (25, 50, 75 e 100 μg/ml) e as possíveis alterações induzidas pelo modelo de endotoxemia (através da injeção intraperitonial de LPS) sobre a atividade de hidrólise de ATP, ADP, AMP e *ρ*-Nph-5'-TMP em plaquetas de ratos machos adultos;

II. Analisar os possíveis efeitos sobre a agregação plaquetária e a contagem de plaquetas em ratos machos adultos endotoxêmicos;

III. Investigar o efeito da injeção intraperitonial de LPS e do agonista específico de adenosina CGS-21680 sobre as atividades de hidrólise dos nucleotídeos da adenina tri- di- e monofosfatados e do substrato artificial *ρ*-Nph-5'-TMP em linfócitos de linfonodos mesentéricos de camundongos machos adultos;

IV. Avaliar as possíveis alterações causadas pelo LPS e CGS-21680 sobre a expressão das ectonucleotidases em linfonodos mesentéricos de camundongos machos adultos;

V. Analisar o efeito da injeção intraperitonial de LPS e do agonista específico de adenosina CGS-21680 sobre as atividades ectonucleotidásicas em membranas renais de camundongos machos adultos;

VI. Verificar as possíveis alterações sobre a expressão gênica das ectonucleotidases de rins de camundongos machos adultos injetados com LPS e CGS-21680;

VII. Avaliar os níveis de nucleotídeos e nucleosídeos extracelulares, por cromatografia liquida de alta eficiência, em membranas renais de camundongos machos adultos expostos ao modelo de endotoxemia e ao CGS-21680.

Material e Métodos

Neste trabalho utilizamos os seguintes modelos:

1. Para a indução de endotoxemia em ratos, e posterior realização de ensaios em plaquetas, foram utilizados ratos machos adultos. O LPS foi injetado intraperitonialmente (i.p.) em uma concentração de 2 mg/kg e os animais foram sacrificados 48 horas após a indução do modelo;

2. Para a indução de endotoxemia em camundongos, o LPS foi injetado i.p. em uma concentração de 12 mg/kg. O CGS-21680 foi administrado em dois momentos, concomitante ou 24 horas após o LPS. Os animais foram sacrificados 24 e 48 horas após a indução do modelo e foram realizados ensaios utilizando linfócitos de linfonodos mesentéricos e preparações de membranas renais.

Artigos Científicos

Capítulo 1:

Endotoxemia altera a hidrólise de nucleotídeos em plaquetas de ratos

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ORIGINAL ARTICLE

Endotoxemia alters nucleotide hydrolysis in platelets of rats

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Abstract

Platelets play a critical role in homeostasis and blood clotting at sites of vascular injury, and also in various ways in innate immunity and inflammation. Platelets are one of the first cells to accumulate at an injured site, and local release of their secretome at some point initiate an inflammatory cascade that attracts leukocytes, activates target cells, stimulates vessel growth and repair. The level of exogenous ATP in the body may be increased in various inflammatory and shock conditions, primarily as a consequence of nucleotide release from platelets, endothelium and blood vessel cells. An increase of ATP release has been described during inflammation and this compound presents proinflammatory properties. ADP is a nucleotide known to induce changes in platelets shape and aggregation, to promote the exposure of fibrinogen-binding sites and to inhibit the stimulation of adenylate cyclase. Adenosine, the final product of the nucleotide hydrolysis, is a vasodilator and an inhibitor of platelet aggregation. There is a group of ecto-enzymes responsible for extracellular nucleotide hydrolysis named ectonucleotidases, which includes the NTPDase (nucleoside triphosphate diphosphohydrolase) family, the NPP (nucleoside pyrophosphatase/phosphodiesterase) family and an ecto-5′-nucleotidase. Therefore, we have aimed to investigate the effect of lipopolysaccharide endotoxin from *Escherichia coli* on ectonucleotidases in platelets from adult rats in order to better understand the role of extracellular adenine nucleotides and nucleosides in the maintenance of blood homeostasis in inflammatory processes. LPS administered in vitro was not able to alter the ATP, ADP, AMP and ρ-Nph-5′-TMP hydrolysis of platelets from untreated rats in all concentrations tested (25-100 μg/ml). There was a significant decrease in ATP, ADP, AMP and ρ-Nph-5′-TMP hydrolysis in rat platelets after 48 hours of LPS exposure (2 mg/Kg, i.p.). ATP and ADP hydrolysis has been reduced about 28% whereas it has been observed a significant 30% and 26% decrease on AMP and ρ-Nph-5′-TMP hydrolysis. Platelet aggregation and platelet number have shown a significant decrease in LPS-treated rats (40% and 55%, respectively) when compared to control group. These results suggest that changes observed in platelet count and, consequently, in nucleotidase activities from circulatory system could alter extracellular nucleotide and nucleoside levels, which might modulate the inflammatory process.

Keywords: *Lipopolysaccharide, nucleoside triphosphate diphosphohydrolase, ATP, 5′-nucleotidase, nucleotide pyrophosphatase/phosphodiesterase, platelets*

Introduction

Platelets are enucleated cell fragments released from megakaryocytes, known to play a major role in the maintenance of endothelial integrity and haemostasis [1]. Platelet function can be seen as a succession of overlapping events involving adhesion, aggregation, secretion and promotion of procoagulant activity [2, 3]. Platelets play a critical role in homeostasis and blood clotting at sites of vascular injury and therefore are important in the development of thrombosis [4]. Platelet aggregation and fibrin deposition can also occur significantly within injured vasculature as a consequence of inflammation [5]. Systemic inflammation is a potent prothrombotic stimulus. Inflammatory mechanisms upregulate procoagulant factors,

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downregulate natural anticoagulants and inhibit fibrinolytic activity. Besides modulating plasma coagulation mechanisms, inflammatory mediators appear to increase platelet reactivity. However, in vivo, natural anticoagulants not only prevent thrombosis, but they also dampen inflammatory activity [6]. Endotoxin, tumor necrosis factor α (TNF-α) and interleukin-1a (IL-1a) induce tissue factor expression, primarily on monocytes/macrophages [7, 8] and probably play a role in inducing tissue factor in atherosclerotic plaques as well [9].

Adenine nucleotides and nucleosides may play a role in the regulation of vascular tone and in platelet aggregation, since ATP and ADP are vasoactive and platelet-active nucleotides, respectively [10, 11]. The level of exogenous ATP in the body may be increased in various inflammatory and shock conditions, primarily as a consequence of nucleotide release from platelets, endothelium and blood vessel cells [12–14]. ADP is a nucleotide known to induce changes in platelets shape and aggregation, to promote the exposure of fibrinogen-binding sites and to inhibit the stimulation of adenylate cyclase [11]. Adenosine, the final product of the nucleotide hydrolysis, is a vasodilator [15] and an inhibitor of platelet aggregation [16]. Several studies have shown the high adenosine levels during inflammatory events or sepsis [17–20].

The most relevant ecto-enzymes involved in adenine extracellular nucleotide hydrolysis are nucleoside triphosphate diphosphohydrolase (NTPDase), nucleotide pyrophosphatase/phosphodiesterase (NPP) families and ecto-5′-nucleotidase (CD73). NTPDase family catalyzes the hydrolysis of γ- and β-phosphate residues of nucleosides 5′-tri and 5′-diphosphates [21]. The family of NPPs is known to hydrolyze 5′-phosphodiester bonds in nucleotides and their derivatives, resulting in the release of 5′-monophosphates [22]. The ecto-5′-nucleotidase catalyzes the extracellular conversion of AMP to the purine nucleoside adenosine [23]. These enzymes are present on the surface of intact platelets and are able to promote the nucleotide hydrolysis in the extracellular space [24–26]. Adenine nucleotides act on platelets via three purinergic receptors: $P2X_1$, $P2Y_1$ and $P2Y_{12}$ receptor [27]. The nucleoside adenosine can mediate its signalling through four G-protein-coupled adenosine receptors named A_1, A_{2A}, A_{2B}, and A_3 [28].

Therefore, we aimed to investigate the effect of lipopolysaccharide endotoxin from *Escherichia coli* on ectonucleotidases in platelets from adult rats in order to better understand the role of extracellular adenine nucleotides and nucleosides in the maintenance of blood homeostasis in inflammatory processes.

Methods

Chemicals

LPS (from *Escherichia coli*, serotype 0111:B4), nucleotides (ATP, ADP and AMP), ρ-Nph-5′-TMP, Malachite Green Base and Coomassie Brilliant Blue G were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Animals

In all experiments, male Wistar rats, approximately 60-90 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 hours 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 (25, 50, 75 and 100 µg/mL) were tested on nucleotide hydrolysis from rat platelets. LPS was preincubated with reaction medium during 10 min and, immediately after, the enzyme assays were performed.

In vivo *experiments*

Rats received one single intraperitoneal injection (1 ml/Kg body weight) with either LPS (2 mg/kg body weight) or saline (control group) [29]. The animals were euthanized 48 hours after injection.

Platelet aggregation assays

The blood for platelet aggregation assays was collected by cardiac puncture from anesthetized (sodium thiopental 40 mg kg^{-1}) adult male rats into plastic tubes containing sodium citrate (3.8%, w/v). Blood samples were centrifuged at $200 \times g$ for 15 min at room temperature to achieve the platelet-rich plasma (PRP) suspension. Platelet aggregation assays were performed on a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA), as previously described [30] with further modifications. Briefly, platelet agonists (10 µM ADP or 2.0 µg/mL collagen), 2.0 mM $CaCl_2$ and Tyrode/BSA were mixed in 96-well-flat-bottom plates. Aggregation was triggered by the addition of 100 µL of platelet suspension in a final reaction volume of 150 µL. The plate was incubated for 2 min at 37°C before the beginning of stirring and readings were followed at 650 nm every 11 s for 20 min. Changes in turbidity

were measured in absorbance units and the results were obtained as area under the aggregation curves. Platelet aggregation and shape change were also monitored using an aggregometer (Chrono-Log Co., Havertown, PA, USA).

Platelet count

The blood for platelet count was collected by cardiac puncture from anesthetized (sodium thiopental $40\,mg\,kg^{-1}$) adult male rats into vacutainer plastic tube containing sodium citrate. Blood samples were analysed in a Coulter JT counter. The data are expressed as the number of platelets $(mm^3)^{-1}$.

Platelet isolation

For platelet isolation, about 5.0 mL of blood was collected in plastic tubes containing sodium citrate (3.8%, w/v). The blood was centrifuged at $200 \times g$ for 5 min. The supernatant containing white blood cells was then centrifuged at $200 \times g$ for 30 min to generate platelet rich plasma (PRP). Resting platelets were then isolated exactly as previously described by Hangtan [12]. Briefly, platelets were separated from plasma by gel filtration on a 1.5×7.0 cm Sepharose 2B column [24]. The column was equilibrated with a buffer consisting of 140 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 5.5 mM dextrose, 0.2 mM EGTA and 0.05% (w/v) sodium azide, pH 6.8 (Ca^{2+}-free Tyrode's buffer). Platelets were eluted with the same buffer at room temperature. Fractions containing about 0.5 mL of platelets were collected and the tubes containing the maximum platelet content (visually determined) were used for subsequent experiments. Polyethylene or siliconized labware was used for all platelet isolation and incubation procedures.

Enzyme assays

Measurement of platelets ATP, ADP, and AMP hydrolysis. Unless otherwise stated, the reaction medium used to assay Ca^{2+}-ATPase and Ca^{2+}-ADPase activity contained 120 mM NaCl, 5.0 mM KCl, 60 mM glucose, 5.0 mM $CaCl_2$ and 50 mM *Tris*–HCl buffer, pH 7.5, in a final volume of 200 µL. About 20 µg of platelet preparation was added to the reaction medium and pre-incubated for 10 min at 37°C. The enzyme reaction was started by the addition of ATP or ADP to a final concentration of 0.5 mM. Incubation times and protein concentrations were chosen to ensure the linearity of the reaction. The reaction was stopped by the addition of 200 µL of 10% trichloroacetic acid (TCA). The amount of Pi liberated was carried out as previously outlined [31]. Controls to correct for non-enzymatic hydrolysis were prepared by adding platelet preparations after the reaction was stopped with TCA. All samples were run in triplicate. Enzyme activities were expressed as nmol Pi released per min per mg of protein. For the ecto-5′-nucleotidase assay, we used the same procedure and conditions, except that ATP and ADP as substrates were replaced by AMP (0.5 mM final concentration) and 5.0 mM $CaCl_2$ was replaced by 5.0 mM $MgCl_2$. The other procedures were the same as those for ATP and ADP hydrolysis.

Measurement of platelets ρ-Nph-5′-TMP hydrolysis. ρ-Nph-5′-TMP (ρ-5′-timidine monophosphate) hydrolysis was determined essentially as described by Furstenau et al. [26]. The reaction medium used to assay ρ-Nph-5′-TMP activity contained 120 mM NaCl, 5.0 mM KCl, 60 mM glucose, 0.5 mM $CaCl_2$ and 50 mM Tris–HCl buffer, pH 8.9, in a final volume of 200 µL. About 20 µg of platelet preparation was added to the reaction medium and preincubated for 10 min at 37°C. The enzyme reaction was started by the addition of ρ-Nph-5′-TMP (to a final concentration of 0.5 mM) at 37°C for 80 minutes 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 activity was expressed as nanomoles (nmol) of ρ-nitrophenol released per minute per milligram of protein.

Protein determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard [32].

Statistical analysis

Data were analysed by Student's T test or one-way ANOVA, followed by the Tukey multiple range test. $P < 0.05$ was considered to represent a significant difference in the statistical analysis used.

Results

In vitro *effect of LPS on ectonucleotidase activities*

The direct effect of LPS upon ectonucleotidase activities has been tested by incubating platelet samples obtained from untreated animals with different LPS concentrations (25, 50, 75 and 100 µg/mL). LPS tested in vitro was not able to alter the ATP, ADP, AMP and ρ-Nph-5′-TMP hydrolysis of platelets from untreated rats in all concentrations tested (Figure 1a–b).

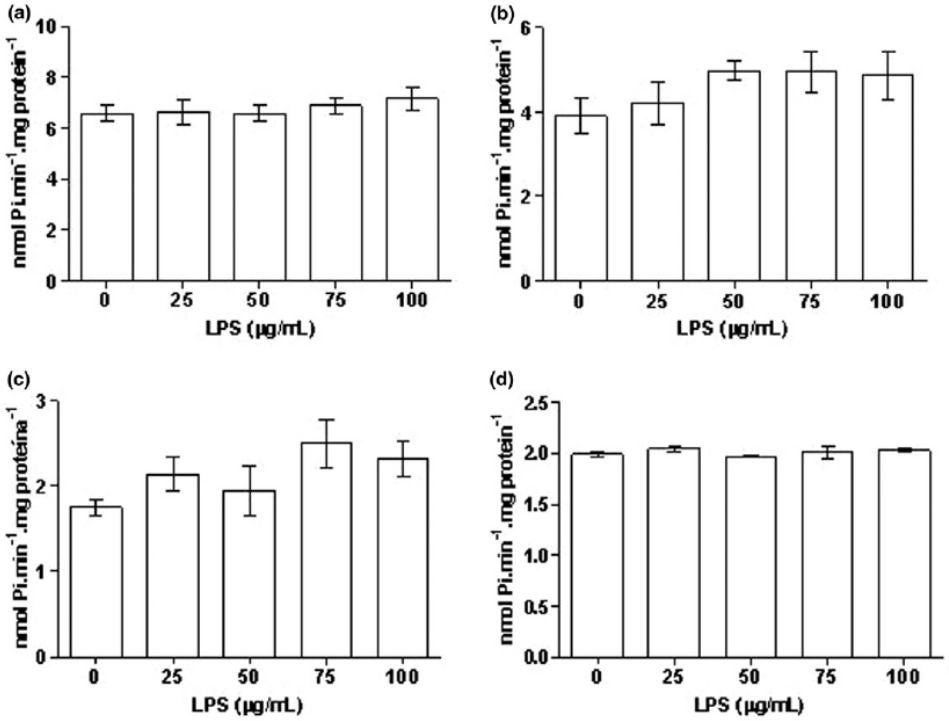

Figure 1. Influence of different concentrations of LPS (25, 50, 75 and 100 µg/ml) on ATP (a), ADP (b), AMP (c) and ρ-Nph-5′-TMP (d) hydrolysis in a preparation rich in platelets of rats. The control activities in platelets were 6.78 ± 0.70, 3.70 ± 0.54 and 1.75 ± 0.18 nmol $Pi.min^{-1}.mg^{-1}$ of protein for ATP, ADP and AMP, respectively. The control of specific activity in platelets was 1.99 ± 0.04 nmol ρ-nitrophenol.min^{-1}.mg^{-1} of protein. The data represent a mean ± SEM ($n = 5$ at least). Statistical analysis were performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test, considering $P < 0.05$ as significant.

Effect of endotoxemia model on ectonucleotidase activities in platelets

There was a significant decrease in ATP, ADP, AMP and ρ-Nph-5′-TMP hydrolysis in rat platelets after 48 hours of LPS exposure. ATP and ADP hydrolysis has been reduced about 28% (Figures 2a and 2b, respectively) whereas it has been observed a significant 30% and 26% decrease on AMP and ρ-Nph-5′-TMP hydrolysis (Figures 2c and 2d, respectively).

Effect of endotoxemia model on platelet function

Figure 3 shows the effect of LPS injection on ADP and collagen induced platelet aggregation. Results (means ± S.E.) have been expressed as percentage of maximal aggregation of four replicates for each group. The control group has been considered as 100% of platelet aggregation. The result obtained shows a significant decrease in platelet aggregation (40%) when collagen has been used as agonist (Figure 3). This difference has been also observed when using aggregometer to assess the platelet function (data not shown).

Platelet count of blood samples from rats injected with LPS

In Figure 4 we tested if LPS administration was able to alter platelet count. There was a significant decrease in platelets count of rats injected with LPS (55%) when compared to the control group.

Discussion

These findings have shown that the endotoxin model is able to alter extracellular nucleotide hydrolysis in rat platelets after 48 hours of LPS administration (Figure 2). Our results have shown a significant effect on nucleotidase activities after in vivo exposure, but LPS per se was not able to alter the nucleotide hydrolysis (Figure 1), which suggest that the changes observed are a consequence of inflammatory response.

Platelets play a role in the process of haemostasis, in various ways in innate immunity, and inflammation. It also contains numerous secretory products and can exert critical roles in several aspects of haemostasis. Platelets may recognize several types of infectious pathogens and limit microbial colonization by sequestering these pathogens and releasing

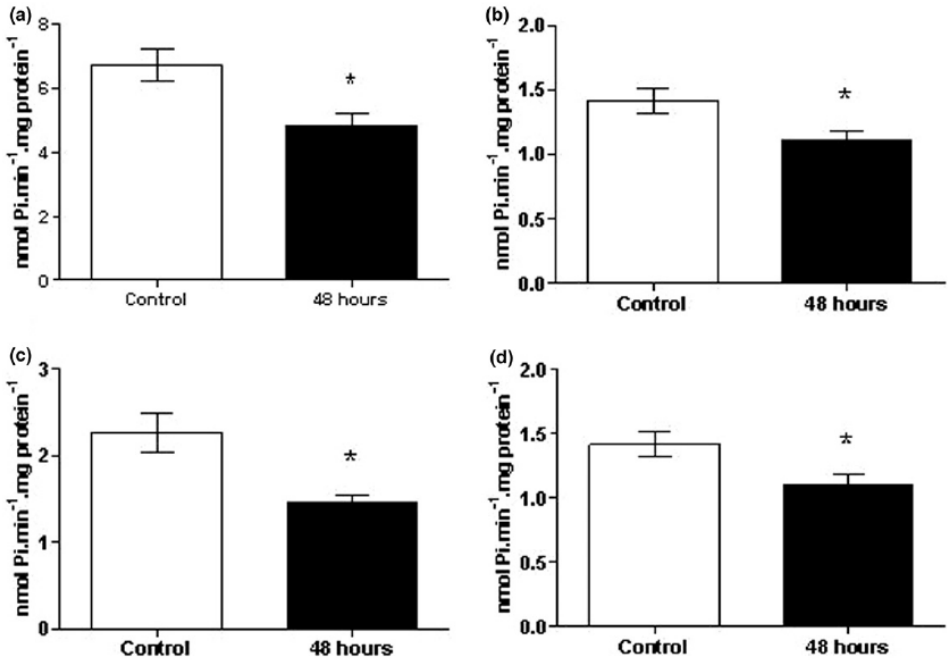

Figure 2. ATP (a), ADP (b), AMP (c) and ρ-Nph-5′-TMP (d) hydrolysis in a preparation rich in platelets of rats after 48 hours of endotoxemia induction. The control of specific activities in platelets were 6.71 ± 1.21, 4.24 ± 0.65 and 2.10 ± 0.53 nmol Pi.min^{-1}.mg^{-1} of protein for ATP, ADP and AMP, respectively. The control of specific activity in platelets was 1.42 ± 0.21 nmol ρ-nitrophenol.min^{-1}.mg^{-1} of protein. The data represent a mean ± SEM ($n = 5$ 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 (*).

immunomodulatory factors [33]. Platelets are one of the first cells to accumulate at an injured site, and local release of their secretome at some point initiate an inflammatory cascade that attracts leukocytes, activates target cells and stimulates vessel growth and repair [34]. Adding to the complexity of the inflammatory process is the growing acceptance that platelets are inflammatory cells that have functional roles beyond stanching blood loss and repairing vascular injury. Furthermore, platelets regulate a variety of inflammatory responses and are key effectors in inflammatory syndromes including atherosclerosis, cancer and sepsis [35, 36].

ATP and its metabolites, ADP and adenosine, at low concentrations (in the micromolar range) influence vascular tone, cardiac function and platelet aggregation [37, 38]. An increase of ATP release has been described during inflammation and this compound has presented proinflammatory properties [39]. The ecto-enzymes responsible for the extracellular hydrolysis of these nucleotides are the NTPDases and NPPs. The NTPDases are capable of hydrolyzing nucleoside tri and/or diphosphates, but not monophosphates [23]. Namely, eight different *E-NTPD* genes encode members of the NTPDase protein family, with four of the NTPDases (NTPDase1, 2, 3 and 8) being expressed as cell surface-located enzymes. All cell-surface members of NTPDase family are highly glycosylated proteins showing close immunological cross-reactivity [40]. The NPP family consists of seven structurally related ecto-enzymes and only the first three members of this family, NPP1-3 are relevant in the context of the purinergic signaling cascade [22, 41]. Ecto-5′-nucleotidase, otherwise known as CD73, is the final step of the extracellular nucleotide breakdown cascade and efficiently hydrolyses 5′-AMP but shows no activity towards nucleoside 2′- and 3′-monophosphates [42]. It has been demonstrated that ecto-5′-nucleotidase found on the surface of endothelial cells converts AMP to adenosine and inhibits platelet aggregation in vitro [43]. Treatment of human blood with soluble 5′-nucleotidase inhibits platelet aggregation via adenosine production and *in vivo* treatment with 5′-nucleotidase has inhibit aggregation and haemostasis [4].

The results observed in Figure 2 have shown a decrease of ATP, ADP, AMP and ρ-Nph-5′-TMP after induction of endotoxemia model. Such changes in nucleotidase activities suggest that there are altered nucleotide and nucleoside levels in extracellular medium. Since ADP acts as an aggregating factor [11] and adenosine inhibits platelet aggregation [16], we have tested if lipopolysaccharide administration has altered platelet coagulation in this model of endotoxemia. The results obtained have demonstrated that there is a decrease in platelet aggregation when collagen has been used

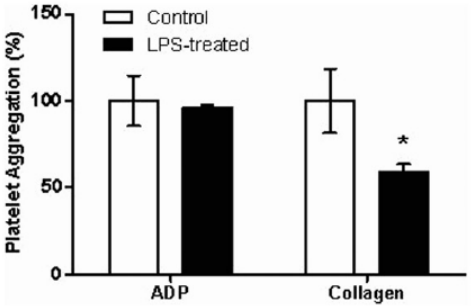

Figure 3. Platelet aggregation in a preparation rich in platelets of rats after 48 hours of endotoxemia induction. ADP and collagen were used to induce platelet aggregation. Results (mean ± SEM) are expressed as percentage of maximal aggregation of four replicates for each group. The control group was considered as 100% of platelet aggregation. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a Tukey multiple range test, considering $P < 0.05$ as significant (*).

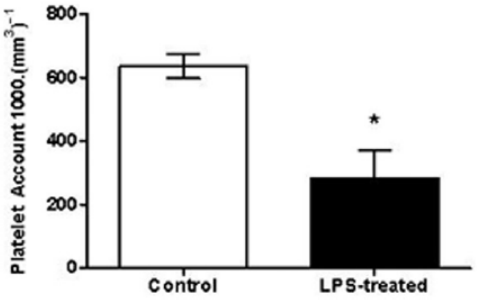

Figure 4. Platelet count in blood of rats after 48 hours of LPS injection. The control of platelet count was 635.875 ± 108.179. The data represent a mean ± SEM ($n = 8$) of number of platelets $(mm^3)^{-1}$. Statistical analyses was performed by test T, considering $P < 0.05$ as significant (*).

as agonist (Figure 3). To better investigate the influence of inflammation in platelet function we have tested if LPS administration could alter platelet count. The results obtained in Figure 4 corroborate with the results observed in Figures 2 and 3, that have shown a significant decrease on ATP (Figure 2a), ADP (Figure 2b), AMP (Figure 2c) and ρ-Nph-5′-TMP (Figure 2d) hydrolysis, besides the decrease in platelet aggregation when collagen has been used as agonist (Figure 3).

Results obtained in our group have demonstrated a significant increase on nucleotide hydrolysis at 24 and 48 hours after exposure to LPS [44]. 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. Vuaden et al. [44] have also shown a decrease on nucleotide hydrolysis after LPS exposure in rat blood serum. The results observed on nucleotide hydrolysis from blood serum are similar to the results that are presented in this paper. The nucleotidases could control the circulating nucleotide levels and present an important role in the maintenance of normal physiology. Since nucleotides exert different responses in diverse tissues, it is noteworthy that cells can co-express two or more enzymes for nucleotide hydrolysis, indicating that they may present different catalytic properties and may perform distinct physiological functions. Multiple physiological roles for E-NPPs have been related, including nucleotide recycling and modulation of purinergic receptor signaling [22]. Previous studies have reported that cells and tissues can co-express distinct ectonucleotidases that share common characteristics [23–26, 41, 42]. The enormous molecular diversity and overlapping tissue distribution of the members of the E-NTPDase and E-NPP families make it difficult to assign specific functions to these enzymes in individual tissues [23]. However, since these enzymes present different kinetic properties, they can act under distinct physiological conditions and can be differently regulated. By converting ADP, released from aggregated platelets, to AMP, the platelet E-NTPDase may play an important role in the prevention of microthrombus formation [24]. In addition, phosphodiesterases may act as 'guard dogs' to prevent subversion of the cell by destroying incoming DNA or RNA [45].

In summary, these findings indicate that there is a tendency to decrease nucleotidase activities and platelet function in rats exposed to endotoxemia model. Such changes could alter extracellular nucleotide and nucleoside levels in circulatory system, which can modulate the inflammatory events.

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Capítulo 2:

Agonista do receptor A2A de adenosina (CGS-21680) previne os efeitos induzidos por LPS nas atividades nucleotidásicas em linfócitos de camundongos

Manuscrito Submetido ao Periódico "European Journal of Pharmacology"

Adenosine A2A receptor agonist (CGS-21680) prevents endotoxin-induced effects on nucleotidase activities in mouse lymphocytes

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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 specific agonist of the adenosine A2A 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 reversed for ATP, adenosine 5’-monophosphate (AMP), and p-Nitrophenyl thymidine 5'-monophosphate (p-Nph-5'-TMP) hydrolysis. However, when CGS-21680 was administered 24 hours after LPS injection, the increase was not reversed. The expression pattern of ectonucleotidases was not altered between LPS and LPS+CGS 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 A2A agonists.

Keywords: lipopolysaccharide, lymphocytes, nucleoside triphosphate diphosphohydrolase, nucleotide pyrophosphatase/phosphodiesterase, 5´-nucleotidase, adenosine.

1. Introduction

Inflammation is a complex homeostatic mechanism devised to protect the integrity of the organism against endogenous or exogenous noxious agents (Medzhitov, 2008). Molecules of microbial origin, such as lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, have long been known to exert an adjuvant effect on the adaptive immune response (Condie et al., 1968).

In recent years, the role of purinergic signaling in the regulation of immune and inflammatory responses has become more evident. An increase in ATP release during inflammation has been described and this compound presents proinflammatory properties (Bodin and Burnstock, 1998). Adenosine, a neuromodulator in the central and peripheral nervous system, is a nucleoside formed by the enzymatic breakdown of ATP. It has been reported that tissue damage and inflammation are accompanied by an accumulation of extracellular adenosine due to its release from non-immune and immune cells (Sitkovsky, 2003). Adenosine interacts with at least four different G-coupled receptors: A1, A2A, A2B, and A3 (Fredholm et al., 2001). A role for this purine nucleoside in the control of inflammation has been suggested due to its anti-inflammatory properties, acting mainly on adenosine A2A receptors (Sullivan, 2003; Thiel et al., 2003; Capecchi et al., 2005). Furthermore, it has been proposed that the administration of adenosine A2A agonists could be useful in inflammatory events and sepsis (Thiel et al., 2003; Sullivan et al., 2004).

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 in regulating the duration and magnitude of purinergic signaling. A wide variety of enzymes are involved in the control of extracellular nucleotide and nucleoside levels:

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). 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 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. 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, 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 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 specific agonist of the adenosine A2A receptor (CGS-21680) on ectonucleotidase activities in lymphocytes from mice submitted to an endotoxemia model. Furthermore, we evaluated the E-NTPDase, E-NPP, and ecto-5´-nucleotidase expression in mesenteric lymph nodes in order to better understand the involvement of extracellular nucleotide hydrolysis in this process.

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 grams) 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 hours 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 ml/kg body weight. Mice were randomly divided in: (i) control group (S), who received a single saline injection; (ii) CGS group (C), who received a single injection of CGS-21680; (iii) LPS group (L24), which was submitted to the endotoxemia model by a single injection of LPS; and (iv) LPS+CGS (LC24), 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 48 hours group (L48), which was submitted to the endotoxemia model by a single injection of LPS and 24 hours later received a single injection of saline; and (vi) LPS+CGS (LC48), which was submitted to the endotoxemia model by a single injection of LPS and 24 hours later received a single injection of CGS-21680. All animals were euthanized by decapitation 24 hours 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 minutes. 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) 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 7.5, in a final volume of 200 μl. Approximately 10^6 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 minutes at 37 ºC. The reaction was stopped by the addition of 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 minute per 10^6 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 10^6 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 minutes 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 X 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 minute per 10^6 cells (Sakura et al., 1998; Vuaden et al., 2009).

2.7 Analysis of gene expression by semi-quantitative RT-PCR

Analysis of the expression of NTPDase1 (Entpd1), 2 (Entpd2), 3 (Entpd3), 8 (Entpd8), NPP1 (Enpp1), 2 (Enpp2), 3 (Enpp3), and 5'-nucleotidase (Nt5e) was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Twenty-four and/or 48 hours 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 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 (SE). 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 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 CGS-21680 and endotoxemia model on ectonucleotidase activities in lymphocytes

After 24 hours of LPS exposure (L24) we observed a significant increase in ATP hydrolysis (178%) when compared with the control group. This increase was reversed in the presence of CGS-21680 (LC24). Likewise, in the L48 group we observed a significant increase in ATP hydrolysis (135%) when compared with control, although this was not reversed by the administration of CGS-21680 (LC48) (Fig. 1A). Figure 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. The hydrolysis of the artificial substrate, *ρ*-Nph-5'-TMP, used to determine phosphodiesterase activities, was significantly increased in the L24 group when

compared to control (62%). This increase was reversed in the presence of CGS-21680 (LC24). The L48 group also presented a significant increase (116%) in *ρ*-Nph-5'-TMP hydrolysis when compared to saline-treated controls (Fig. 2A), although this increase in hydrolysis persisted in LC48. For AMP hydrolysis we observed a significant increase in the L24 group (207%) when compared to the control group, which returned to control levels in the LC24 group. Similarly, in the L48 group a significant increase in AMP hydrolysis was also observed (121%), and with CGS-21680 administration (LC48) this increase was once again maintained, when compared with control (Fig. 3A).

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

The expression patterns after CGS and LPS treatment are presented in Figures 1C, 1D (for Entpd1, 2, 3), 2B, 2C (for Enpp1, 2, 3), 3B, and 3C (for Nt5e). The results show that mRNA transcript levels were altered in LPS and LPS+CGS groups when compared to saline group for Entpd3 and Enpp3, predominantly. For others enzyme mRNA transcripts we did not observed significant alterations in mesenteric lymph nodes after the treatments. 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 significant changes in ectonucleotidase activities in lymphocytes of mice after LPS and/or CGS-21680 exposure.

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 A2A receptors (Di Virgilio et al., 2009).

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).

In our results, we observed a significant increase in nucleotide hydrolysis in lymphocytes after mice had been exposed to endotoxemia, both at 24 hours and 48 hours after LPS exposure (groups L24 and L48). 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 specific agonist of A2A receptor, on nucleotide catabolism. When CGS-21680 was administered alone, we did not observe a significant difference compared to the control group (injected i.p. with saline). However, when CGS-21680 was administered concomitantly with LPS, the increase in nucleotide hydrolysis promoted by LPS exposure was reversed for ATP, AMP, and 5'-TMP hydrolysis (LC24 group, Figures 1A, 3A, and 2A, respectively). Despite this effect, when CGS-21680 was administered 24 hours after LPS injection, the increase was not reversed (LC48 group), which lead us to the hypothesis that this A2A agonist can prevent the effect of inflammation on ectonucleotidase activities, but cannot reverse this effect when the endotoxemia model has already been established. Despite the effects observed on ATP hydrolysis, there was no significant difference between LPS and LPS+GCS groups for ADP hydrolysis. Ectonucleotidases present different abilities to hydrolyze nucleotides tri and di-phosphates and NTPDase2, also known as ecto-ATPase and CD39L1, has a high preference for nucleoside triphosphates (30:1 ATP/ADP hydrolysis ratio) (Zimmermann, 2001). A probable reason for the results observed in this study is that NTPDase2 is affected by the CGS-21680 only in the initial stages of inflammatory process. Moreover, the expression pattern of ectonucleotidases presents an increase in mRNA levels in groups treated with LPS and LPS plus CGS mainly for Entpd3 and Enpp3. For other enzyme mRNA transcript levels analyzed, the differences between treated groups and control group were not so evident, indicating that the changes in ectonucleotidase activities were also a consequence of transcriptional control. In contrast, in the LC24 group, in spite of the increase in mRNA transcripts for some ectonucleotidases analyzed, the nucleotide hydrolysis remained near to control values.

The transcriptional control was not involved in the preventive effect exerted for CGS-21680. Therefore, such effect may occur due to phosphorylation control, since these enzymes present potential phosphorylation sites on their extracellular domains (http://www.cbs.dtu.dk, NetPhosk, a tool for the kinase-specific prediction of protein phosphorylation sites).

Adenosine receptors are coupled to G-proteins, and adenosine A2A and A2B receptors in particular can increase intracellular cAMP levels by activating adenylate cyclase. The expression pattern of adenosine receptor subtypes (A1, A2A, A2B, and A3) varies depending on the cell type and pharmacological and biochemical studies have established that A2A 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 A2A signaling pathways are active in limiting inflammation and tissue injury (Haskó and Cronstein, 2004; Linden, 2005; Sitkovsky and Ohta, 2005; Haskó and Pacher, 2008). The interaction between adenosine and A2A receptors is capable of inhibiting inflammation by cAMP induction (Ohta and Sitkovsky, 2009). Experimental data suggest that A2A agonists and antagonists can mediate inflammation by activating and blocking, respectively, an A2A-dependent immunomodulatory mechanism (Ohta and Sitkovsky, 2001). A2A 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 (Haskó et al., 2000; Pinhal-Enfield et al., 2003; Lappas et al., 2005). Specific A2A agonists have been tested in many different inflammation models, and the results show that these new compounds are successful in the

suppression of inflammatory disease states (Odashima et al., 2005; Lappas et al., 2006; Sevigny et al., 2007).

A study performed by Deaglio and colleagues (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 A2A-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 hours before CGS-21680, this compound failed to reduce the effect of LPS. Probably, in the initial phase of inflammation the activation of A2A receptor was able to control the ectonucleotidase stimulation. In contrast, when the inflammatory process has been already established and the production of adenosine has been enhanced, the role of CGS-21680 on ectonucleotidase activities control was not effective.

5. Conclusion

In summary, these results indicate that there is an enhancement of extracellular nucleotide catabolism in lymphocytes after the induction of the endotoxemia model,

which was prevented, but not reversed by CGS-21680 administration. These results suggest that the control of nucleotide and nucleoside levels exerted by CGS-21680 could be exploited to modulate the effects of A2A agonists during the inflammatory process.

Disclosure/Conflict of interest

The authors report no conflicts of interest.

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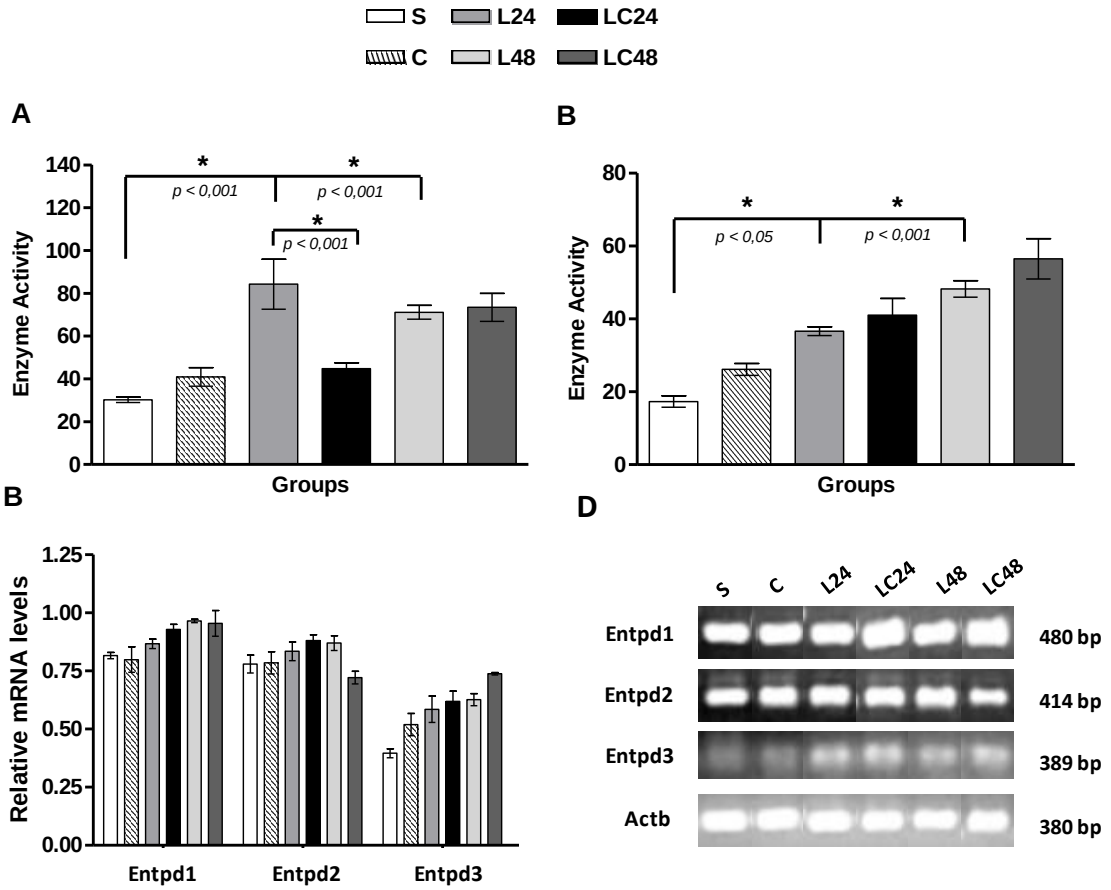
Legends to Figures

Fig. 1. ATP (A) and ADP (B) hydrolysis in lymphocytes from mesenteric lymph nodes of mice 24 and 48 hours after endotoxemia induction and CGS-21680 treatment. The control values for enzyme activities in lymphocytes from treated animals were 30.22 ± 1.14 and 17.31 ± 1.56 nmol $Pi.min^{-1}.10^{-6}$ cells for ATP and ADP, respectively. The data represent mean ± SE (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 (*). Figures C and D represent gene expression patterns of Entpd1, Entpd2, Entpd3 and Actb in mesenteric lymph nodes of mice. Mice were injected with LPS and/or CGS-21680 and after 24 or 48 hours of exposure the mesenteric lymph nodes were excised. Total RNA was isolated and subjected to RT-PCR for the indicated targets. Three independent experiments were performed, with entirely consistent results.

Fig. 2. ρ-Nph-5'-TMP hydrolysis (A) in lymphocytes from mesenteric lymph nodes of mice 24 and 48 hours after endotoxemia induction and CGS-21680 treatment. The control value for enzymatic activity of ρ-Nph-5'-TMP in lymphocytes from treated animals was 2.81 ± 0.15 nmol ρ-nitrophenol.$min^{-1}.10^{-6}$ cells. The data represent a mean ± SE (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 (*). Figures B and C represent gene expression patterns of Enpp1, Enpp2, Enpp3 and Actb in mesenteric lymph nodes of mice. Three independent experiments were performed, with entirely consistent results.

Fig. 3. AMP hydrolysis (A) in lymphocytes from mesenteric lymph nodes of mice 24 and 48 hours after endotoxemia induction and CGS-21680 treatment. The control value for enzymatic activities in lymphocytes from treated animals was 1.65 ± 0.11 nmol $Pi.min^{-1}.10^{-6}$ cells. The data represent a mean ± SE (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 (*). Gene expression patterns of Nt5e and Actb in mesenteric lymph nodes of mice are represented in Figures B and C. Three independent experiments were performed, with entirely consistent results.

Figure 1:


S
L24
LC24
C
L48
LC48
A
Enzyme Activity
Groups
p < 0,001
p < 0,001
p < 0,001
B
Enzyme Activity
Groups
p < 0,05
p < 0,001
B
Relative mRNA levels
Entpd1
Entpd2
Entpd3
D
S
C
L24
LC24
L48
LC48
Entpd1
Entpd2
Entpd3
Actb
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414 bp
389 bp
380 bp

Figure 2:

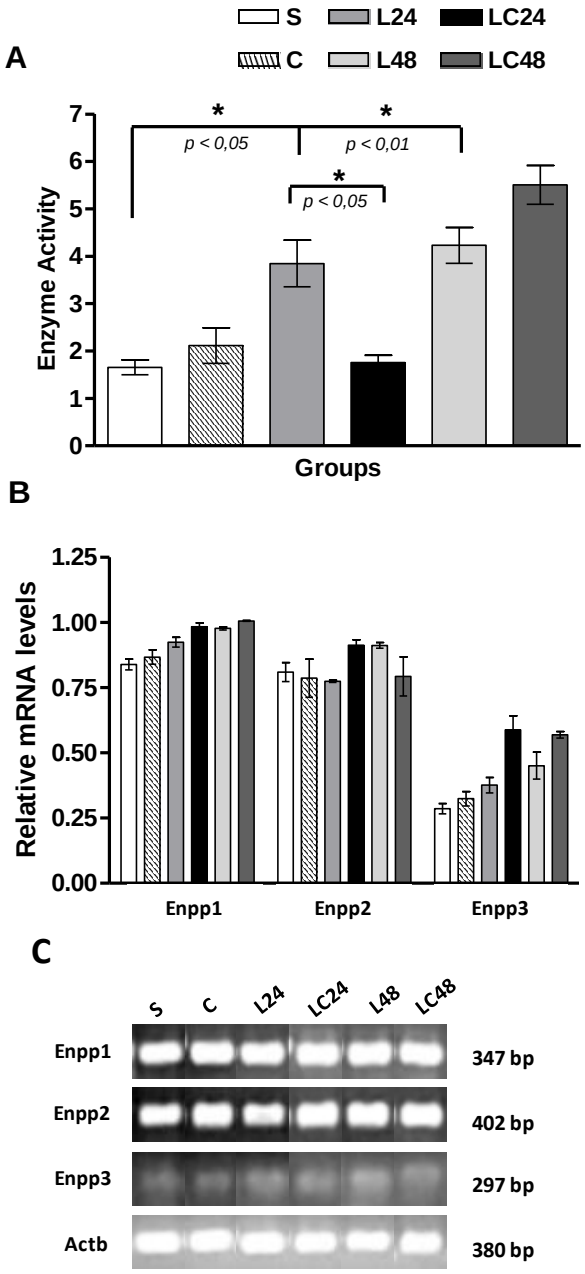
A
S
L24
LC24
C
L48
LC48
*
p < 0,05
*
p < 0,01
*
p < 0,05
Enzyme Activity
Groups
B
Relative mRNA levels
Enpp1
Enpp2
Enpp3
C
S
C
L24
LC24
L48
LC48
Enpp1
Enpp2
Enpp3
Actb
347 bp
402 bp
297 bp
380 bp

Figure 3:

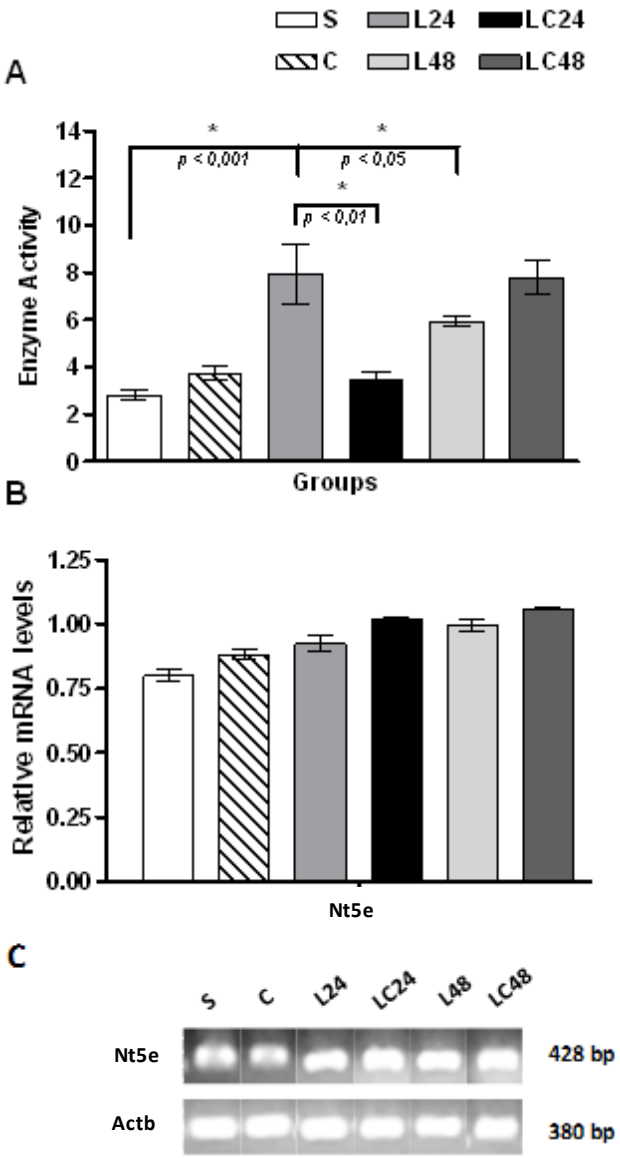
A
S
L24
LC24
C
L48
LC48
Enzyme Activity
p < 0,001
p < 0,05
p < 0,01
Groups
B
Relative mRNA levels
Nt5e
C
S
C
L24
LC24
L48
LC48
Nt5e
428 bp
Actb
380 bp

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

Capítulo 3:

Efeitos induzidos por endotoxina sobre as atividades ectonucleotidásicas em rins de camundongos

Manuscrito em Preparação

Endotoxin-induced effects on ectonucleotidase activities in kidney from mice

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Abstract

Severe acute kidney injury (AKI) is a frequently condition in hospital patients and there is strong evidence that sepsis is an important cause of AKI. Extracellular ATP mainly functions as a proinflammatory mediator. Adenosine, the final product of ATP breakdown, is an anti-inflammatory compound, acting mainly on adenosine A2A receptors. Considering that kidney is an organ strongly affected by inflammation and ectonucleotidases are responsible for the control of extracellular nucleotides and nucleosides levels, we investigated the effect of a specific agonist of the adenosine A2A receptor (CGS-21680) on ectonucleotidase activities and mRNA expression, as well on extracellular nucleotide levels, in kidney membrane preparations from endotoxemic mice. 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 analysis of ectonucleotidase expression was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Extracellular nucleotide and nucleoside levels were measured by HPLC assay. Exposure to endotoxemia promoted an increase in ATP and 5'TMP hydrolysis, and a decrease on AMP hydrolysis. CGS-21680 exposure was not able to reverse these alterations. The expression pattern of ectonucleotidases demonstrated an increase on Entpd3 and Enpp3 mRNA levels after LPS injection, but not for other enzymes analyzed, indicating that the transcriptional control was not the only involved on the effect exerted by LPS. HPLC analysis indicated a decrease on extracellular ATP and adenosine levels in treated groups. These findings indicate that there is an alteration on nucleotide and nucleosides viability in kidney from mice under different moments of endotoxemia model, in order to protect the integrity of this organ exposed to inflammation.

Keywords: kidney, lipopolysaccharide, nucleoside triphosphate diphosphohydrolase, nucleotide pyrophosphatase/phosphodiesterase, 5´-nucleotidase.

Introduction

Severe acute kidney injury (AKI) is a condition that can be shown to occur in almost 8% of hospital patients and in roughly 50% of patients in Intensive Care Unit (ICU) (Hoste et al., 2006; Uchino et al., 2006). Moreover, around 5% of ICU patients with AKI require acute renal replacement therapy (Uchino et al., 2005). Therefore, AKI is a significant clinical problem in modern hospitals. There is strong evidence that sepsis and septic shock are the most important causes of AKI in critically ill patients and correspond to 50% or more of cases of AKI in ICU (Silvester et al., 2001; Hoste et al., 2006).

Inflammation is an adaptive response that is produced by 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, acts activating TLR4 receptors and inducing inflammatory response (Alving, 1993; Akira et al., 2001).

Extracellular nucleotides and nucleosides play important roles during inflammatory process. Extracellular ATP mainly functions as a proinflammatory and immunostimulatory mediator in the microenvironment of damage cells (Bours et al., 2006). ATP activates family of P2 receptors, that comprises P2Y G-protein coupled receptors and P2X receptors, which are ligand-gated ion channels (Ralevic and Burnstock, 1998). The acceptance that extracellular ATP participates in inflammation increased after P2 receptors were cloned and their expression in inflammatory cells was completely characterized (Di Virgilio, 2009). P2 receptors are expressed in the kidney and are found mainly in glomeruli and tubules (Unwin et al., 2003; Bailey et al., 2004).

Adenosine, the final product of ATP breakdown, is an important signaling molecule and adenosine receptors are recognized as important molecular targets in the pathophysiology of inflammation (Blackburn et al., 2009). Adenosine interacts with P1 receptors family that comprises four different G-coupled receptors subtypes (A1, A2A, A2B, and A3) (Klotz, 2000; Fredholm et al., 2001). A role for this nucleoside in the control of inflammation has been suggested due to its anti-inflammatory properties, acting mainly on adenosine A2A receptors (Sullivan, 2003; Thiel et al., 2003; Capecchi et al., 2005). A2A 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 (Haskó et al., 2000; Lappas et al., 2005).

The control of extracellular nucleotide and nucleoside levels occur through the action of ectonucleotidases. This group of ectoenzymes is composed by the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the ecto-pyrophosphatase/phosphodiesterase (E-NPP) family and the ecto-5'-nucleotidase (EC 3.1.3.5) (Zimmermann, 2001). Specifically, eight different *ENTPD* genes encode members of the NTPDase protein family, with four of the NTPDases (NTPDase1, 2, 3, and 8) being expressed as cell surface-located enzymes (Zimmermann, 2001, Bigonnesse et al., 2004). The E-NPP family consists of seven structurally related ectoenzymes; however, only NPP1-3 are capable to hydrolyze various nucleotides and are relevant in the context of the purinergic signaling cascade (Goding et al., 2003, Yegutkin, 2008). Ecto-5'-nucleotidase is the enzyme responsible for AMP hydrolysis, generating the nucleoside adenosine (Zimmermann, 1996) and have also been proposed to present 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 kidney is an organ strongly affected by inflammation and ectonucleotidases are responsible for the control of extracellular nucleotides and nucleosides levels, which play an important role during inflammatory events, we investigated the effect of a specific agonist of the adenosine A2A receptor (CGS-21680) on ectonucleotidase activities and mRNA expression in kidney membrane preparations from mice submitted to an endotoxemia model. We also evaluated the changes in extracellular nucleotide levels in order to better understand the involvement of extracellular nucleotide hydrolysis in this process.

Materials and methods

Chemicals

CGS-21680, Lipopolysaccharide (LPS) from *Escherichia coli*, serotype 0111:B4, Coomassie Blue, 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). Trizol® Reagent, 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.

Animals

In all experiments, male F1 mice (approximately 8-10 weeks old, weighing around 50 grams) 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 hours 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.

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 ml/kg body weight. Mice were randomly divided in: (i) control group (S), who received a single saline injection, (ii) CGS group (C), who received a single injection of CGS-21680, (iii) LPS group (L24), which was submitted to the endotoxemia model by a single injection of LPS, and (iv) LPS+CGS (LC24), 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 (L48), which was submitted to the endotoxemia model by a single injection of LPS and 24 hours later received a single injection of saline, and (vi) LPS+CGS (LC48), which was submitted to the endotoxemia model by a single injection of LPS and 24 hours later received a single injection of CGS-21680. All animals were euthanized by decapitation 24 hours after the last injection.

Kidney Membranes Preparation

Kidney membranes were prepared essentially as previously outlined by Nagy and Delgado-Escueta (1984) with minor modifications. Briefly, about 1.0 g of both right and left kidneys were dissected on ice, washed, and gently homogenized in 10 volumes of medium containing 0.32 M sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.5 (Medium I), and then centrifuged at 1000 *g* during 10 minutes. The supernatant was collected and centrifuged again at 12000 *g* for 20 minutes. The 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 a centrifugation at 15000 *g* for 20 minutes, the fractions that banded at the 10%/16% Percoll interface were collected with wide-tip disposable plastic transfer pipette. The kidney membrane fraction was washed twice with Medium I by centrifugation at 15000 *g* for 20 minutes to remove the contaminating Percoll. The pellet from the second centrifugation was resuspended to a final concentration of 0.5 - 0.8 mg/mL. The membranes were prepared fresh daily and maintained at 0 - 4ºC throughout the experimental procedure.

Protein Determination

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

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, 2.0 μg/mL oligomycin, pH 7.5, in a final volume of 200 μL. The ADP hydrolysis was assessed using the same reaction mixture described above, except by the fact that 1.0 mM $CaCl_2$ was used 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 were added per tube and were preincubated for 10 minutes at 37 °C. The enzyme reactions were started by the addition of nucleotide as substrates in a final concentration of 1.0 mM (ATP/ADP) or 2.0 mM (AMP). After 10 minutes 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 a previous study (Vieira et al., 2001). The amount of inorganic phosphate (Pi) released was carried out using a colorimetric method as previously outlined by Chan and colleagues (1986). Controls to correct for non-enzymatic substrate hydrolysis were performed by adding membrane fractions after the reactions had been stopped with TCA. All samples were performed in triplicate. Enzyme activities were generally expressed as nmol Pi released per minute per milligram of protein.

Assay of Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) Activity

The phosphodiesterase activity was assessed using *p*-Nph-5'-TMP (an artificial substrate). Briefly, for assay of kidney membranes 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, 1.5 mM $CaCl_2$, pH 8.9, was preincubated with approximately 13 μg per tube of kidney membrane protein for 10 minutes 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 minutes of incubation time, 200 μL 0.2 N 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 X 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 samples were performed in triplicate. Enzyme activity was generally expressed as nmol *p*-nitrophenol released per minute per milligram of protein (Sakura et al., 1998).

Analysis of gene expression by semi-quantitative RT-PCR

Analysis of the expression of NTPDase1, 2, 3, 8 (Entpd1, 2, 3, 8), NPP1, 2, 3 (Enpp1, 2, 3), 5'-nucleotidase (Nt5e) was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Twenty-four and/or 48 hours after treatments, both right and left kidney of mice (n=3 for each group) were removed for total RNA extraction with Trizol® Reagent 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, 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, 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 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.

Analysis of purine levels in kidney membrane preparations by high pressure liquid chromatography (HPLC)

The samples were incubated with 100 μM of ATP. The denaturation of sample proteins was performed using 0.6 mol/L perchloric acid. All samples were then centrifuged (14000 *g* for 10min) and the supernatants were neutralized with 4.0 N KOH and clarified with a second centrifugation (14000 *g* for 15 min). Aliquots of 40 μL were applied to a reverse-phase HPLC system using a 25 cm C_{18} Shimadzu column (Shimadzu, Japan) at 260 nm with a mobile phase containing 60 mM KH2PO4, 5.0 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol according to a method previously described (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. The results are expressed as nmoles of the different compounds per μg of protein in the incubation time. All incubations were carried out in triplicate and the

controls to correct non-enzymatic hydrolysis of nucleotides were performed by measuring the peaks present into the same reaction medium incubation without kidney membrane. The control for kidney membrane secretion of purines was performed by incubation the kidney membrane without the substrate under the same conditions described above.

Statistical analysis

Results are expressed as means ± standard error (SE). 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 $P < 0.05$.

Results

Effect of CGS-21680 and endotoxemia model on ectonucleotidase activities in kidney membranes

After 48 hours of LPS exposure (L48) we observed a significant increase in ATP hydrolysis (48%; $P<0.05$) when compared with the control group. When we administered CGS-21680 24 hours after LPS exposure (LC48) we observed a trend to an increase in ATP hydrolysis, but it was not significant when compared with the control group. For L24 and LC24 groups there was no significant difference when compared to the control (Fig. 1A). Figure 1B shows ADP hydrolysis after LPS induction and CGS-21680 exposure. There was no significant difference between groups that received LPS or LPS plus CGS-21680 and the control groups.

The hydrolysis of the artificial substrate, *ρ*-Nph-5'-TMP, used to determine E-NPP activities, was significantly increased in the L48 (47%; $P<0.05$) and LC48 (51%;

P<0.05) groups when compared to saline-control group. This increase was not observed in L24 and LC24 groups (Fig. 2A).

For AMP hydrolysis, we observed a significant decrease in the L24 group (40%; P<0.05) and with CGS-21680 administration (LC24) (37%; P<0.05) when compared to the control group. In the L48 and LC48 groups there was no significant difference in AMP hydrolysis when compared with control (Fig. 3A). When we injected CGS only, it was not able to alter enzymatic activities when compared to saline for all nucleotides tested.

Effect of CGS-21680 and LPS on ectonucleotidases mRNA expression in kidney

The gene expression patterns of ectonucleotidases after CGS-21680 and LPS treatment were evaluated and the results show that Entpd3 (Fig.1C and 1D), Enpp1 and Enpp3 (Fig 2B and 2C) transcript levels were increased in LC48 groups. Likewise L48 groups that demonstrated an increase in expression pattern of Entpd3 and Enpp3. For Nt5e, we did not observe alterations in mRNA transcripts for the groups tested (Fig. 3B and 3C).

Purine level measurement

The purine levels in the kidney membrane preparations of control and treated groups were measured by HPLC (Table 2). The results showed that all treated groups presented a decrease on extracellular ATP levels, higher o L48 and LC48 groups. For extracellular ADP levels, we did not observe significant difference between control and treated groups. For AMP, after 3 minutes of incubation, L48 and LC48 groups indicated an accumulation on extracellular levels of this nucleotide. After 10 minutes of incubation, this accumulation was more apparently in L24 and LC24 groups. The

adenosine extracellular levels demonstrated a decrease in all groups treated when compared to control.

Discussion

In the present study, we observed significant changes on ectonucleotidase activities in kidney membrane preparations of mice after LPS and/or CGS-21680 exposure, as well as in mRNA transcript levels of these ecto-enzymes.

The involvement of extracellular ATP in inflammation has been demonstrated since 1970 and 1980 decades (Dahlquist and Diamant, 1970; Cockcroft and Gomperts, 1980). These molecules appear to be "endogenous signal" and exhibit chemotactic and activating effects on leukocytes, displaying potent immuno-enhancing activity (Oppenheim & 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 receptor and several works demonstrated the participation of ATP in inflammatory process, acting via this purinoceptor subtype (Solle et al., 2001; Labasi et al., 2002; Le Feuvre et al., 2002; Elliott & Higgins, 2004).

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 exposed to 48 hours of endotoxemia model when compared control group. For ADP hydrolysis, there was a profile to increase in the same group, but it was not statistically significant. The hydrolysis of 5'-TMP, an artificial substrate using to determine NPP activity, as observed for ATP, was also increased in L48 group. These results could be related to a compensatory response after 48 hours of LPS injection, decreasing ATP availability,

and its proinflammatory properties. Similar results on ATP and ADP hydrolysis were observed by our group when we tested if 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 that AMP hydrolysis demonstrated a different pattern of variation in kidney membrane preparations. We showed a significant decrease on AMP hydrolysis after 24 hours of LPS injection and there was no difference on AMP hydrolysis between L48 and control group. It is possible to suggest that AMP hydrolysis in L48 group returns to control values due to the stoichiometric effect promoted by the increase in the other nucleotide hydrolysis. 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 (Cunha, 2001).

Similar to ATP, adenosine can also be considered as a danger molecule because its extracellular levels rise markedly in response to tissue damage. However, contrasting with ATP, elevated extracellular adenosine levels mediate an autoregulatory immunosuppressive loop to protect healthy tissues (Bours et al., 2006). The four members of P1 receptors are G protein coupled. A1 and A3 receptors are coupled to Go/Gi protein, causing inhibition of adenylyl cyclase and, consequently, decreasing the production of cAMP (32-36 - Guan). Conversely, A2A and A2B receptors are coupled to Gs protein, leading to stimulation of adenylyl cyclase, increasing cAMP levels (37, 38 - Guan). A2A 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 in activating pro-resolution pathways (Bours et. al, 2006; Haskó and Pacher, 2008). A2A receptors in kidney have been detected in the renal microvasculature (Okusa, 2002), as well as on mesangial (Sholz-Pedretti et al., 2001) and tubular epithelial cells (Lee and Emala, 2002). For this

reason, we tested if CGS-21680, a specific agonist of adenosine A2A receptor, promotes different modulations on ectonucleotidase activities in kidney membrane preparations of endotoxemic mice. The CGS-21680 was administered in two different ways, concomitant with LPS (LC24 group) or 24 hours after LPS injection (LC48 group). The changes observed in nucleotide hydrolysis in kidney membrane preparations of mice during endotoxemia model were not reversed by CGS-21680 administration. When compared to saline group, AMP hydrolysis was significant decreased on LC24 group, 5'TMP hydrolysis was significant increased on LC48 group, and ATP hydrolysis maintain a profile of increase, but not statistically significant.

In relation of ectonucleotidases expression, there were an increase in Entpd3 and Enpp3 mRNA transcript levels after 48 hours of LPS exposure and, when CGS-21680 was administered 24 hours after LPS (LC48 group), this increase was observed in Entpd3, Enpp1 and Enpp 3 expression levels. Probably, the alterations observed in ATP and 5'TMP hydrolysis is indicating that changes observed in ectonucleotidase activities were also a consequence of transcriptional control. In contrast, we did not observe alterations in Nt5e mRNA levels for the groups tested. One possible reason for the changes observed in ecto-5'-nucleotidase activities, and also for the other ecto-enzymes, is the phosphorylation control, since ectonucleotidases present potential phosphorylation sites on extracellular domains (Bigonesse et al., 2004; Lavoie et al., 2004) (http://www.cbs.dtu.dk, NetPhosk, a tool for the kinase-specific prediction of protein phosphorylation sites).

Purinoceptors and ectonucleotidases expression in immune cells change under inflammatory conditions, allowing for the progressive acquirement of an immunomodulatory purinergic repertoire expressed by the cells involved in inflammatory and immune responses. The ecto-enzymes ENTPDase1 and ecto-5'-

nucleotidase (also identified as CD39 and CD73) control extracellular nucleotide levels and thus regulate the extent of purinergic signaling (Bours et al., 2006). Here we reported that LPS administration induces alterations on ectonucleotidases activities in kidney membrane preparations of mice. The decreased AMP hydrolysis after 24 hours of endotoxemia model lead us to support that, in the initial phase of inflammatory process, the production of extracellular adenosine via AMP was diminished in kidney. However, the ATP and 5'TMP hydrolysis increased when this process was more established (after 48 hours of LPS injection), indicating that the supply of extracellular ATP is reduced. The purine metabolism analysis 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 mechanism for the loss of glomerular filtration rate is that the glomerulus display afferent and efferent arteriolar vasodilatation, but the efferent vasodilatation is higher (Bellomo et al., 2008). Since adenosine is a vasodilator (Kitakase et al., 1991) and ATP acts as a proinflamatory agent (Pelegrin et al., 2008), our results lead us to speculate that there is a reduction on adenosine and ATP availability in kidney during inflammatory events in order to protect this organ of the effects promoted by these molecules.

In summary, our results indicate that there is an alteration on nucleotide and nucleosides viability in kidney from mice under different moments of endotoxemia model, in order to protect the integrity of this organ exposed to inflammation.

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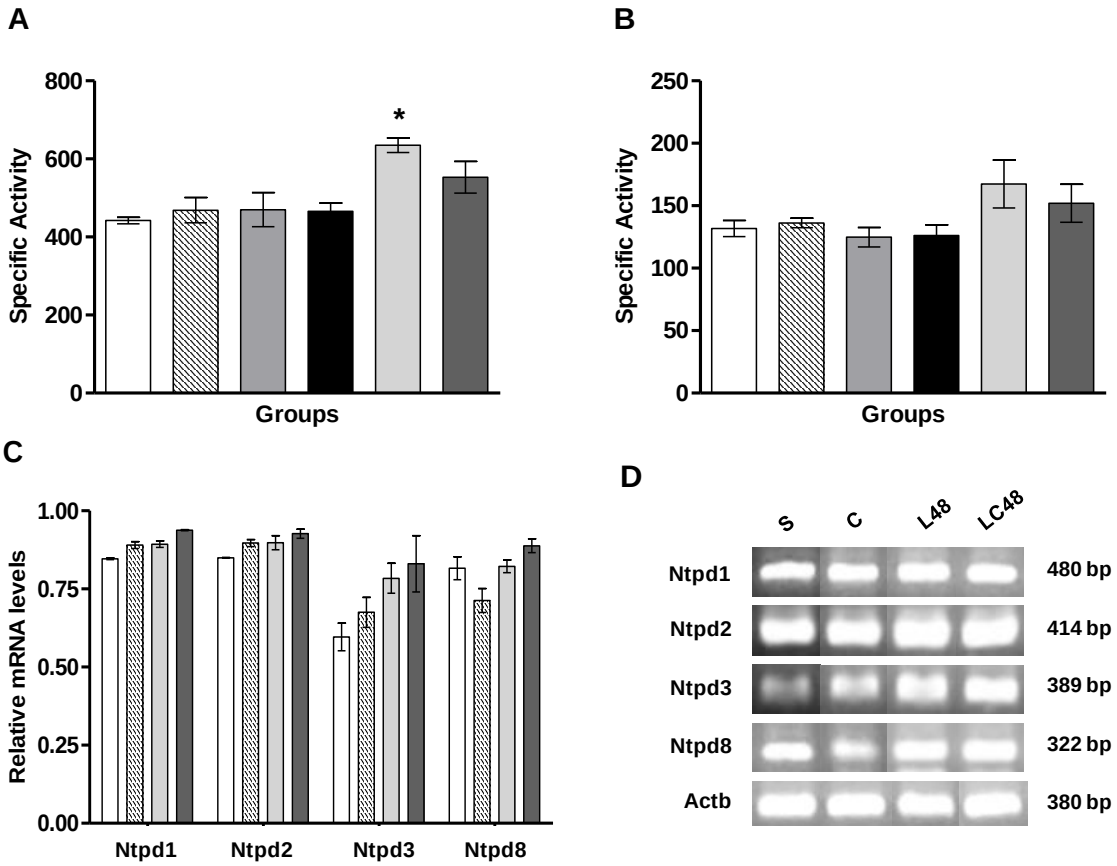
Legends to Figures

Fig. 1. ATP (A) and ADP (B) hydrolysis in kidney membranes preparation of mice 24 and 48 hours after endotoxemia induction and CGS-21680 treatment. The control values for specific activities in kidney membranes preparation from treated animals were 448.38 ± 35.09 and 131.72 ± 14.55 nmol $Pi.min^{-1}.mg^{-1}$ of protein for ATP and ADP, respectively. The data represent mean ± SE (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 (*). Figures C and D represent gene expression patterns of Entpd1, Entpd2, Entpd3 and Actb in kidneys of mice. Mice were injected with LPS and/or CGS-21680 and after 24 or 48 hours of exposure the kidneys were excised. Total RNA was isolated and subjected to RT-PCR for the indicated targets. Three independent experiments were performed, with entirely consistent results.

Fig. 2. ρ-Nph-5'-TMP hydrolysis (A) in Kidney membranes preparation of mice 24 and 48 hours after endotoxemia induction and CGS-21680 treatment. The control value for specific activity of ρ-Nph-5'-TMP in kidney membranes preparation from treated animals was 76.9 ± 2.55 nmol ρ-nitrophenol.$min^{-1}.mg^{-1}$ of protein. The data represent a mean ± SE (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 (*). Figures B and C represent gene expression patterns of Enpp1, Enpp2, Enpp3 and Actb in kidneys of mice. Three independent experiments were performed, with entirely consistent results.

Fig. 3. AMP hydrolysis (A) in kidney membranes preparation of mice 24 and 48 hours after endotoxemia induction and CGS-21680 treatment. The control value for specific activities in kidney membranes preparation from treated animals was 133.76 ± 19.92 nmol $Pi.min^{-1}.mg^{-1}$ of protein. The data represent a mean ± SE (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 (*). Gene expression patterns of Nt5e and Actb in kidneys of mice are represented in Figures B and C. Three independent experiments were performed, with entirely consistent results.

Figure 1:


A
Specific Activity
800
600
400
200
0
*
Groups
B
Specific Activity
250
200
150
100
50
0
Groups
C
Relative mRNA levels
1.00
0.75
0.50
0.25
0.00
Ntpd1
Ntpd2
Ntpd3
Ntpd8
D
S
C
L48
LC48
Ntpd1
Ntpd2
Ntpd3
Ntpd8
Actb
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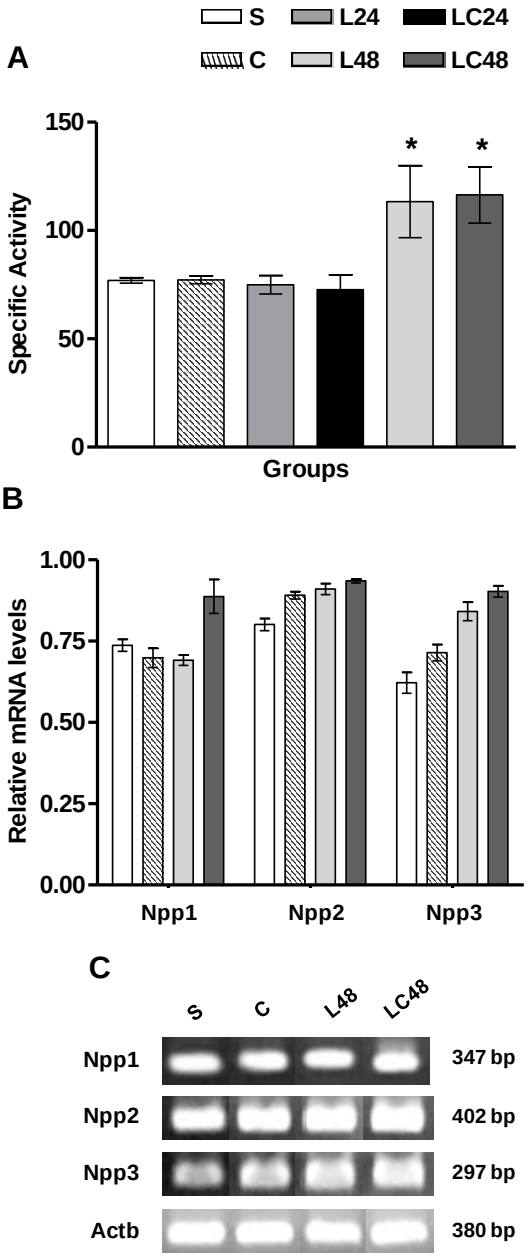
389 bp
322 bp
380 bp

Figure 2:


A
S
L24
LC24
C
L48
LC48
Specific Activity
150
100
50
0
*
*
Groups
B
Relative mRNA levels
1.00
0.75
0.50
0.25
0.00
Npp1
Npp2
Npp3
C
S
C
L48
LC48
Npp1
Npp2
Npp3
Actb
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297 bp
380 bp

Figure 3:

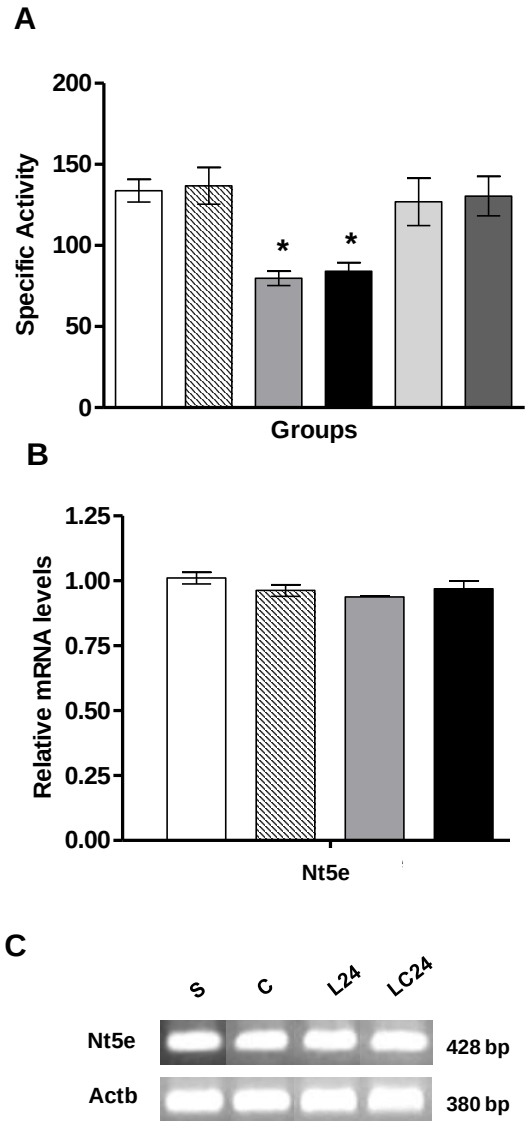
A
200
150
100
50
0
Specific Activity
*
*
Groups
B
1.25
1.00
0.75
0.50
0.25
0.00
Relative mRNA levels
Nt5e
C
S
C
L24
LC24
Nt5e
428 bp
Actb
380 bp

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

Table 2: In vitro effect of LPS and CGS-21680 on extracellular nucleotide and nucleoside levels from kidney membrane preparations

Purines	Time point	Groups					
		SAL	**CGS**	**L24**	**LC24**	**L48**	**LC48**
ATP	0	3.650	3.650	3.650	3.650	3.650	3.650
	3	2.400	1.420	1.750	0.960	0.910	1.160
	10	1.760	1.270	0.620	0.160	0.167	0.115
	30	0.840	0.580	0.270	0.090	0.157	0.102
	60	0.590	0.196	0.150	0.103	0.107	0.093
	120	0.550	0.050	0.125	0.008	0.021	0.005
	180	0.370	0.000	0.098	0.003	0.005	0.003
ADP	0	0.090	0.090	0.090	0.090	0.090	0.090
	3	1.370	1.070	1.330	1.160	2.090	1.340
	10	0.890	0.870	0.910	0.300	0.643	0.440
	30	0.660	0.450	0.340	0.350	0.280	0.230
	60	0.410	0.450	0.200	0.103	0.280	0.180
	120	0.290	0.450	0.300	0.030	0.008	0.031
	180	0.190	0.048	0.230	0.004	0.001	0.006
AMP	0	0.056	0.056	0.056	0.056	0.056	0.056
	3	0.690	0.880	0.870	1.010	1.670	1.670
	10	1.610	1.490	2.410	1.500	2.190	1.650
	30	0.640	0.400	1.130	1.010	0.560	0.510
	60	0.230	0.400	0.730	0.610	0.380	0.480
	120	0.210	0.246	0.008	0.043	0.019	0.230
	180	0.033	0.016	0.002	0.003	0.009	0.023
Adenosine	0	0, 000	0, 000	0, 000	0, 000	0, 000	0, 000
	3	0.330	0.500	0.121	0.220	0.210	0.320
	10	1.220	1.200	0.980	0.970	0.790	0.940
	30	1.410	1.750	1.420	1.920	1.350	1.890
	60	2.140	1.740	1.590	2.180	2.370	2.910
	120	2.560	2.830	2.030	2.230	2.300	1.760
	180	2.680	2.580	1.690	1.940	1.850	1.520

Results are presented as representative of means at least 3 independent experiments for each substrate tested. Standard errors are least of 10%

3. Discussão

As ectonucleotidases são compostas pelas famílias das E-NTPDases e E-NPPs, que hidrolisam os nucleotídeos trifosfatados e difosfatados até seus respectivos nucleotídeos monofosfatados. Por sua vez, a ecto-5'-nucleotidase hidrolisa os nucleotídeos monofosfatados até seus respectivos nucleosídeos (Zimmerman, 2001, Yegutkin, 2008). Essas ecto-enzimas estão ancoradas na membrana celular, possuindo seu sítio catalítico voltado para o meio extracelular (Zimmermann, 2001) e são distribuídas em uma ampla variedade de tecidos e células; sendo que a NTPDase1 e a 5'-nucleotidase são também conhecidas como antígeno de ativação celular linfóide (CD39) e proteína de superfície de linfócitos (CD73), respectivamente.

Através da ativação de receptores purinérgicos do tipo P2X e P2Y, os nucleotídeos da adenina induzem uma série de efeitos pró-inflamatórios (Ia Sala et al., 2003; Burnstock, 2006; Ferrari et al., 2006; Di Virgilio, 2007), enquanto a adenosina exerce seus efeitos anti-inflamatórios principalmente através da ativação do subtipo A2A dos receptores P1 (Haskó e Cronstein, 2004; Sitkovsky e Otha, 2005; Hashó e Pacher, 2008). Da mesma forma que os receptores purinérgicos medeiam os efeitos imunológicos dos nucleotídeos e dos nucleosídeos extracelulares, as ectonucleotidases medeiam a cascata purinérgica que leva a um progressivo decréscimo nas concentrações de nucleotídeos com consequente aumento nos níveis de nucleosídeos (Bours et al., 2006)

Portanto, neste estudo avaliamos as atividades ectonucleotidásicas em diferentes frações e tipos celulares após a indução de endotoxemia em modelos animais, bem como o envolvimento dos receptores A2A na modulação dessas atividades enzimáticas

no intuito de obter maiores esclarecimentos sobre o papel exercido por essas enzimas durante o processo inflamatório.

As plaquetas são células sanguíneas especializadas que exercem um papel central na fisiologia e patofisiologia de processos homeostáticos, inflamatórios, de cicatrização de feridas e crescimento tumoral (Jurk e Kehrel, 2005). As plaquetas são as principais efetoras da homeostase celular e manutenção da integridade endotelial em humanos e outros mamíferos. Apesar das plaquetas serem mais bem conhecidas como mediadores primários da homeostase, o reconhecimento da sua importância durante eventos inflamatórios, atuando na imunidade inata e adquirida, tem aumentado (Weyrich et al., 2003; Sowa et al., 2009; Semple e Freedman, 2010). O papel exercido pelas plaquetas na inflamação crônica e aguda inclui a liberação de mediadores pró-inflamatórios, a ativação de moléculas de superfície que possuem funções inflamatórias e a interação com leucócitos e células endoteliais (Elstad et al., 1995; McIntyre et al., 2003).

Os nucleotídeos e nucleosídeos da adenina exercem diferentes efeitos sobre as plaquetas, atuando na regulação do tônus vascular e na agregação plaquetária. O ATP age como uma molécula vasoativa (Burnstock, 1990), enquanto o ADP é um ativador plaquetário (Colman, 1990). O nucleosídeo adenosina, por sua vez, atua como um vasodilatador (Engler, 1991) e um inibidor da agregação plaquetária (Kitakaze et al., 1991). As enzimas responsáveis pela hidrólise dos nucleotídeos da adenina a adenosina estão presentes na membrana de plaquetas e já tiveram sua caracterização descrita (Frasseto et al., 1993; Furstenau et al., 2006). Com base nesses dados, o objetivo do estudo realizado no Capítulo 1 foi determinar se a inflamação poderia promover alterações nas atividades de hidrólise de ATP, ADP, AMP e 5'-TMP, bem como na agregação e função plaquetária de ratos expostos ao modelo de endotoxemia.

Nos sítios inflamatórios há um aumento na liberação de ATP e este composto apresenta propriedades pró-inflamatórias (Bodin e Burnstock, 1998; Piccini et al., 2008). O ATP e seus metabólitos, ADP e adenosina, em concentrações micromolares influenciam o tônus vascular, a função cardíaca e a agregação plaquetária (Ralevic e Burnstock, 1998; Burnstock, 2004). Os resultados apresentados no Capítulo 1 demonstram que a administração de LPS promoveu alterações sobre as atividades ectonucleotidásicas em plaquetas de ratos. Foi observada uma diminuição significativa na hidrólise de todos os nucleotídeos testados, bem como na hidrólise do substrato artificial 5'-TMP, 48 horas após a indução do modelo de endotoxemia. Em estudos prévios, nosso grupo reportou um decréscimo na hidrólise de nucleotídeos em soro de ratos expostos ao LPS (Vuaden et al., 2007). Esses resultados observados na hidrólise dos nucleotídeos em soro são similares aos resultados apresentados no Capitulo 1. As enzimas presentes em soro poderiam atuar em conjunto com as enzimas de plaquetas e da parede vascular a fim de evitar a agregação plaquetária espontânea e a formação de trombos (Frassetto et al., 1993; Yegutkin & Burnstock, 2000; Oses et al., 2004).

Uma vez que a hidrólise dos nucleotídeos apresentou-se diminuída em plaquetas e considerando-se o papel do ADP e da adenosina como um agregante e um inibidor da agregação plaquetária, respectivamente, foi investigado se a inflamação poderia promover alterações também sobre a função plaquetária. Os resultados obtidos demonstraram uma diminuição na agregação plaquetária e na contagem de plaquetas após a indução do modelo de endotoxemia em ratos. Apesar da contagem de plaquetas apresentar-se diminuída nos animais tratados com LPS, a diminuição na hidrólise dos nucleotídeos não pode ser reportada diretamente a esse fato, uma vez que, para a realização dos ensaios enzimáticos, a concentração de proteína foi normatizada.

Os resultados obtidos no Capítulo 1 demonstram que há uma tendência de diminuição das atividades ectonucleotidásicas e função plaquetária em ratos expostos ao modelo de endotoxemia, sugerindo que as alterações observadas são conseqüência da resposta inflamatória. Tais alterações provavelmente influenciam nos níveis de nucleotídeos e nucleosídeos extracelulares no sistema circulatório, os quais poderiam atuar modulando os eventos inflamatórios.

O interesse da comunidade científica sobre a importância do sistema purinérgico na resposta imune tem aumentado muito nos últimos anos, haja vista a quantidade crescente de estudos descrevendo o papel do sistema purinérgico na ativação de diferentes respostas do sistema imune (Sullivan et al., 1999; Ia Sala et al., 2003; Di Virgilio et al., 2005; Sitkovsky e Lukashev, 2005; Sitkovsky e Ohta, 2005; Bours et al., 2006; Bursntock, 2006; Di Virgilio et al., 2009). O ATP é essencialmente descrito como uma molécula que possui atividade pró-inflamatória, atuando principalmente via ativação do receptor ligado a canais iônicos do tipo P2X7 (Bulanova et al., 2005; Ferrari et al., 2007; Pelegrin et al., 2008), enquanto a adenosina apresenta características de molécula imunomodulatória e imunossupressora (Blackburn et al., 2009). Além do papel exercido pelos nucleotídeos e nucleosídeos da adenina na ativação ou imunossupressão do sistema imune, também tem sido identificada a participação das ectonucleotidases como importantes moléculas envolvidas nesse processo. Além dessas ecto-enzimas serem responsáveis pela manutenção dos níveis de nucleotídeos e nucleosídeos extracelulares, a NTPDase1 (CD39) e a ecto-5'-nucleotidase (CD73) também são descritas por apresentarem atividade imunomodulatória (Deaglio et al., 2007; Dwyer et al., 2007).

Estudos prévios realizados pelo nosso grupo demonstraram que o LPS é capaz de alterar as atividades nucleotidásicas em linfócitos e soro de ratos submetidos ao

modelo de endotoxemia (Vuaden et al., 2007). Nesse trabalho, foi demonstrado que a hidrólise dos nucleotídeos foi aumentada em linfócitos de linfonodos mesentéricos 24 e 48 horas após a indução do modelo. Esse aumento nas atividades ectonucleotidásicas observadas após a administração de LPS pode ser resultado de uma resposta ao processo inflamatório, uma vez que resultaria em diminuição na disponibilidade de ATP extracelular e um aumento na geração de adenosina extracelular. Visto que a adenosina possui atividade anti-inflamatória e imunossupressora, atuando principalmente via ativação do receptor A2A de adenosina (Sitkovsky e Ohta, 2005; Haskó e Pacher, 2008), o objetivo central do estudo realizado no Capítulo 2 foi investigar se o agonista específico do receptor A2A de adenosina (CGS-21680) seria capaz de reverter possíveis alterações promovidas pela inflamação (via injeção intraperitonial de LPS) sobre a atividade de hidrólise de nucleotídeos em linfócitos de linfonodos mesentéricos de camundongos.

Para a realização dos experimentos desenvolvidos no Capítulo 2, foi necessário realizar uma padronização das atividades enzimáticas para a hidrólise dos nucleotídeos tri- di- e monofosfatados testados e do substrato artificial 5'-TMP. Todas as condições ideais necessárias à manutenção da linearidade das reações foram previamente testadas, como tempo de incubação, concentração de células, concentração de substrato, concentração de cátions e pH. Essa padronização foi baseada no estudo de Vuaden e colaboradores (2007), com pequenas modificações. A preparação de linfócitos também sofreu modificações para garantir a manutenção da integridade celular, visto que para os ensaios enzimáticos foram utilizadas apenas preparações com no mínimo 90% de viabilidade celular, verificada através de dosagem de LDH e contagem de células com Azul de Trypan. A preparação apresentou-se enriquecida em linfócitos (mais de 95%) e com pouca contaminação por outros tipos celulares que poderiam interferir nos

resultados, uma vez que os nucleotídeos poderiam estar sendo hidrolisados por ecto-enzimas presentes na membrana de outras células.

Adicionalmente, foram realizadas algumas alterações nas condições de incubação descritas por Vuaden e colaboradores (2007) para assegurar melhores condições à reação. Foram determinadas como condições ideais para a atividade enzimática um meio contendo 1 mM de $CaCl_2$ (para ATP, ADP e 5'-TMP) ou 1 mM de $MgCl_2$ (para AMP), 120 mM de NaCl, 5 mM de KCl, 60 mM de glicose, 1 mM de azida sódica, 0,1% de albumina e 20 mM de tampão Hepes, pH 7.5 (para ATP, ADP e AMP) ou 20 mM de tampão Tris, pH 8.9 (para 5'TMP), em um volume final de 200 μl. A concentração ideal de células foi de 10^6 células e a concentração ideal de ATP, ADP e AMP foi de 2 mM de substrato e o tempo de incubação ideal foi de 30 minutos para esses nucleotídeos. Para a hidrólise de 5'-TMP a concentração final de substrato ideal foi de 0,5 mM, a concentração de células foi de 10^6 células e o tempo de incubação determinado foi de 60 minutos.

Quando o CGS-21680 foi administrado concomitantemente ao LPS, observou-se uma diminuição significativa na hidrólise de ATP, AMP e 5'TMP, mas não de ADP. Já quando o GCS-21680 foi administrado 24 horas após a injeção de LPS, o aumento na hidrólise dos nucleotídeos promovido pela endotoxemia não foi revertido. Esses dados indicam que o CGS-21680 é capaz de prevenir os efeitos promovidos pela inflamação sobre as atividades ectonucleotidásicas, mas não é capaz de reverter esse efeito quando o modelo de endotoxemia já está estabelecido.

As ectonucleotidases apresentam diferentes proporções de hidrólise dos nucleotídeos trifosfatados e difosfatados. A E-NTPDase1 hidrolisa ATP e ADP igualmente bem (razão de hidrólise de 1:1) (Heine et al., 1999; Zimmermann, 2001), a E-NTPDase2 hidrolisa o ATP 30 vezes mais que o ADP (Kirley, 1997; Zimmermann,

2001) e a E-NTPDase3 prefere o ATP em relação ao ADP numa razão de hidrólise de aproximadamente 3:1 (Zimmermann, 2001; Lavoie et al., 2004). Uma explicação possível para o efeito diferentemente exercido pelo CGS-21680 sobre a hidrólise de ATP e ADP é que a enzima E-NTPDase2, também conhecida como ecto-ATPase e com uma maior razão de hidrólise para o ATP, foi uma das mais afetadas quando esse composto foi administrado juntamente com o LPS.

Com relação à expressão das ectonucleotidases, observou-se um aumento nos níveis de RNA mensageiro nos grupos tratados tanto com LPS quanto com LPS e CGS-21680 para os transcritos das enzimas Entpd3 e Enpp3. Já para os transcritos de RNA mensageiro das outras enzimas testadas, a diferença entre os grupos tratados e o grupo controle não foram tão evidentes. Esses dados sugerem que há um controle transcricional envolvido no aumento observado sobre a hidrólise dos nucleotídeos no grupo LPS, com relação às enzimas Entpd3 e Enpp3. A expressão aumentada das ectonucleotidases durante eventos inflamatórios já foi demonstrada, principalmente para as enzimas CD39 (NTPDase1) e CD73 (ecto-5'-nucleotidase) (Duarte et al., 2007; Louis et al., 2008; Reutershan et al., 2009; Spanevello et al., 2010). Provavelmente essas enzimas são mais estudadas devido ao seu importante papel como antígeno de ativação celular linfóide e proteína de superfície celular de linfócitos, respectivamente.

Apesar do aumento nos transcritos de RNA mensageiro observados, no grupo que recebeu LPS e CGS-21680 a hidrólise dos nucleotídeos ATP, AMP e do substrato artificial 5'TMP permaneceram próximos aos valores do grupo controle. Fica evidente que, nessa situação, o controle transcricional não foi responsável pelo efeito preventivo exercido pelo CGS-21680. Entretanto, tal efeito pode ter sido resultado de um controle pós-transcricional, como a fosforilação destas enzimas, uma vez que elas apresentam potenciais sítios de fosforilação nos seus domínios extracelulares para enzimas cinases,

como PKA e PKC, por exemplo (Lavoie et al., 2004); como pode ser conferido através de diferentes ferramentas utilizadas para a predição de possíveis sítios protéicos para fosforilação por cinases (como NetPhosk, KinasePhos, Scansite e PredPhospho, por exemplo).

Numerosos estudos têm demonstrado a participação do receptor A2A de adenosina nos mecanismos de sinalização envolvidos no controle da inflamação (Haskó and Cronstein, 2004; Linden, 2005; Sitkovsky and Ohta, 2005; Haskó and Pacher, 2008). Os receptores A2A apresentam numerosas propriedades anti-inflamatórias, como a inibição da ativação de células T (Huang et al., 1997; Erdmann et al., 2005) e o controle na produção de mediadores inflamatórios, como IL-12, TNFα· e INFγ (Haskó et al., 2000; Pinhal-Enfield et al., 2003; Lappas et al., 2005). No estudo realizado no Capítulo 2, foi observado que o efeito do LPS sobre as atividades ectonucleotidásicas foi prevenido quando o LPS foi co-administrado com o agonista específico do receptor A2A (CGS-21680). Entretanto, quando o CGS-21680 foi administrado 24 horas após o LPS, esse agonista não foi capaz de reverter os efeitos apresentados de aumento na hidrólise dos nucleotídeos promovidos pelo LPS. Isso indica que a ativação do receptor A2A é eficiente para controlar a estimulação sobre as ecto-enzimas na fase inicial do processo inflamatório. Em contrapartida, quando o processo inflamatório está estabelecido e a produção de adenosina já está aumentada (devido à estimulação exercida pela indução de endotoxemia sobre as ectonucleotidases), o papel do CGS-21680 no controle das atividades ectonucleotidásicas não demonstra ser efetivo.

A injúria renal aguda (IRA) é uma condição comum em pacientes internados em centros de tratamento intensivo está associada a altas taxas de mortalidade (Uchino et al., 2005; Bagshaw et al., 2007; Dennen et al., 2010), sendo que a sepse severa e o choque séptico são fatores associados aos casos de IRA reportados em pacientes

internados em CTI (Silvester et al., 2001; Toft e Gilsaa, 2007). Apesar do crescente número de estudos sobre tratamentos relacionados a essa patologia, ainda não há conhecimento sobre tratamentos realmente efetivos (Venkataraman, 2008; Rajapakse e Wijewickrama, 2009; Dennen et al., 2010).

Devido ao importante papel exercido pelos nucleotídeos e nucleosídeos extracelulares durante os eventos inflamatórios, e da relação existente entre os casos de inflamação severa e falência renal, no Capítulo 3 foi avaliado se a indução do modelo de endotoxemia em camundongos poderia alterar as atividades ectonucleotidásicas e o padrão de expressão dessas enzimas, bem como os níveis extracelulares de nucleotídeos e nucleosídeos, em preparações de membranas renais. Como a adenosina exerce suas ações muito fortemente via ativação do receptor A2A (Haskó et al., 2000; Haskó e Pacher, 2008), o efeito da administração do agonista específico para esse receptor (CGS-21680) sobre os mesmos parâmetros também foi analisado.

Nos resultados obtidos no Capítulo 3, verificou-se um aumento significativo na hidrólise de ATP e 5'-TMP em membranas renais 48 horas após a indução do modelo de endotoxemia. Para a hidrólise do ADP, foi observado um perfil de aumento, mas que não foi estatisticamente significativo. Esses resultados podem ser relacionados a uma resposta compensatória no rim, exercida através do aumento das atividades ectonucleotídásicas, resultando em diminuição do ATP disponível, no sentido de minimizar seus efeitos inflamatórios. Quando o CGS-21680 foi administrado, tanto concomitante, quanto 24 horas após o LPS, não foi observada nenhuma alteração sobre a hidrólise dos nucleotídeos testados, ou seja, o CGS-21680 não foi capaz de prevenir e/ou reverter os efeitos induzidos pelo modelo endotoxêmico sobre as ectonucleotidases de membranas renais. A hidrólise do ATP e 5'-TMP aumentada em 48 horas indica que

o suprimento de ATP extracelular é diminuído quando o processo inflamatório está em um estágio mais avançado.

Com relação à hidrólise do AMP, os resultados observados foram diferentes daqueles encontrados para os outros substratos testados. Ao contrário de um aumento na hidrólise desse nucleotídeo, o que se observou foi uma diminuição significativa 24 horas após a injeção de LPS. Já após 48 horas de indução do modelo, a hidrólise do AMP parece retornar aos níveis próximos do controle. Uma possível explicação para o aumento na hidrólise do AMP (retornando aos níveis próximos do controle) 48 horas após a injeção de LPS está relacionada com o aumento observado sobre as hidrólises de ATP e 5'TMP nesse mesmo tempo. Esse resultado possibilitaria sugerir que a hidrólise do AMP aumenta devido ao efeito estequiométrico promovido pelo aumento na hidrólise dos outros nucleotídeos, bem como da eficiente remoção destes, reduzindo a inibição exercida pelo ATP (numa razão maior de ATP/ADP) sobre a ecto-5'-nucleotidase, a qual poderia permitir uma maior geração de adenosina (Cunha, 2001).

Para investigar se as alterações das atividades ectonucleotídásicas e o seu padrão de expressão ocasionam mudanças no metabolismo extracelular do ATP, os níveis de compostos purinérgicos produzidos ao longo do tempo (0-180min) foram avaliados por HPLC, a partir da incubação com 100 μM de ATP da preparação de membranas renais do grupo controle e dos grupos tratados com LPS e/ou CGS-21680. O sobrenadante da incubação foi desproteinizado, aplicado no HPLC e os compostos purinérgicos determinados. Todos os grupos tratados apresentaram níveis menores de ATP extracelular em relação ao grupo controle ao longo de todo o tempo de incubação. Em relação aos níveis extracelulares de ADP, não encontramos diferenças significativas entre os grupos analisados. Após 3 minutos de incubação, o grupo injetado 48 horas com LPS e o que recebeu injeção de CGS-21680 24 horas após a de LPS acumularam

AMP, indicando uma menor hidrólise desse substrato em relação aos demais grupos. Esse acúmulo de AMP foi mais aparente nos dois grupos injetados com LPS, 24 e 48 horas após a indução do modelo, após 10 minutos de incubação. Apesar de uma diferença significativa na atividade da ecto-5´-nucleotidase ter sido encontrada apenas nos grupos tratados 24 horas com LPS e que foram co-administrados com LPS e CGS, observou-se uma variação nos níveis de AMP extracelular em todos os grupos tratados ao longo do tempo de incubação, provavelmente em resposta às alterações na concentração do AMP produzido a partir do ATP e do ADP. Finalmente, observou-se um atraso na produção de adenosina a partir do ATP incubado nos grupos tratados, provavelmente em decorrência da redução na hidrólise do AMP.

A diminuição na hidrólise do AMP 24 horas após a indução do modelo de endotoxemia indica que, na fase inicial do processo inflamatório, a produção de adenosina extracelular via AMP foi diminuída no rim. Apesar da importância de uma maior compreensão sobre os mecanismos envolvidos na injúria renal aguda, pouco ainda é conhecido. Uma dos possíveis mecanismos propostos por Bellomo e colaboradores (2008) para a diminuição na pressão capilar glomerular, na taxa de filtração glomerular e na produção de urina observados durante a IRA seria que, durante um evento séptico, a vasodilatação arteriolar eferente é maior do que a aferente. Devido ao papel de molécula vasodilatadora exercido pela adenosina (Kitakase et al., 1991), pode-se sugerir que há uma modulação da atividade da enzima ecto-5'-nucleotidase no sentido de diminuir a disponibilidade de adenosina extracelular no rim durante o processo inflamatório, visando proteger esse órgão dos efeitos vasodilatadores desencadeados por esse nucleosídeo.

Com relação à expressão das ectonucleotidases, foi observado um aumento nos transcritos de RNA mensageiro das Entpd3 e Enpp3 48 horas após exposição ao LPS.

Quando o CGS-21680 foi administrado 24 horas depois do LPS, esse aumento foi observado para os transcritos de Entpd3, Enpp1 e Enpp3. Provavelmente, as alterações observadas na hidrólise de ATP e 5'-TMP ocorreram em decorrência também de controle transcricional. Em contraste, para os transcritos de RNA mensageiro de Nt5e não foi visualizada nenhuma alteração após 24 horas de indução do modelo. Uma razão possível para as alterações observadas nas atividades ecto-5'-nucleotidásicas, e também para as outras ecto-enzimas testadas, seria uma resposta ao controle pós-transcricional via fosforilação, uma vez que as ectonucleotidases apresentam potenciais sítios para fosforilação nos seus domínios extracelulares (Bigonesse et al., 2004; Lavoie et al., 2004).

A expressão de purinoceptores e das ectonucleotidases é alterada em células imunes sob condições inflamatórias, levando a um progressivo ganho no repertório purinérgico nas células envolvidas nas respostas imunes e inflamatórias (Louis et al., 2008; Reutershan et al., 2009; Spanevello et al., 2010). As ecto-enzimas CD39 e CD73 controlam os níveis de nucleotídeos e nucleosídeos extracelulares e regulam a extensão da sinalização purinérgica (Bours et al., 2006). Como observado, os resultados obtidos no Capítulo 3 indicam que há uma alteração na viabilidade dos nucleotídeos e nucleosídeos da adenina em diferentes momentos após a indução do modelo de endotoxemia, atuando no sentido de proteger a integridade desse órgão dos efeitos promovidos por essas moléculas durante a inflamação.

Este trabalho demonstra a interação cruzada entre a ativação dos receptores A2A de adenosina e as enzimas que modulam a produção do agonista desse receptor, o que pode estar envolvido no complexo conjunto de ações que são desencadeadas pelo processo inflamatório. Portanto, a Figura 5 resume as alterações promovidas pela endotoxemia e ativação dos receptores A2A. Os resultados deste trabalho indicam que a

endotoxemia é capaz de modular as atividades ectonucleotidásicas em diferentes frações biológicas, sugerindo que essas enzimas participam da resposta inflamatória. Em linfócitos este efeito foi revertido pela ativação dos receptores A2A. Esses dados indicam que a ativação dos receptores A2A exerce um mecanismo modulatório sobre as ectonucleotidases, sendo que este pode ser um dos mecanismos relacionados com as ações anti-inflamatórias e a resposta imune mediada por esse receptor.

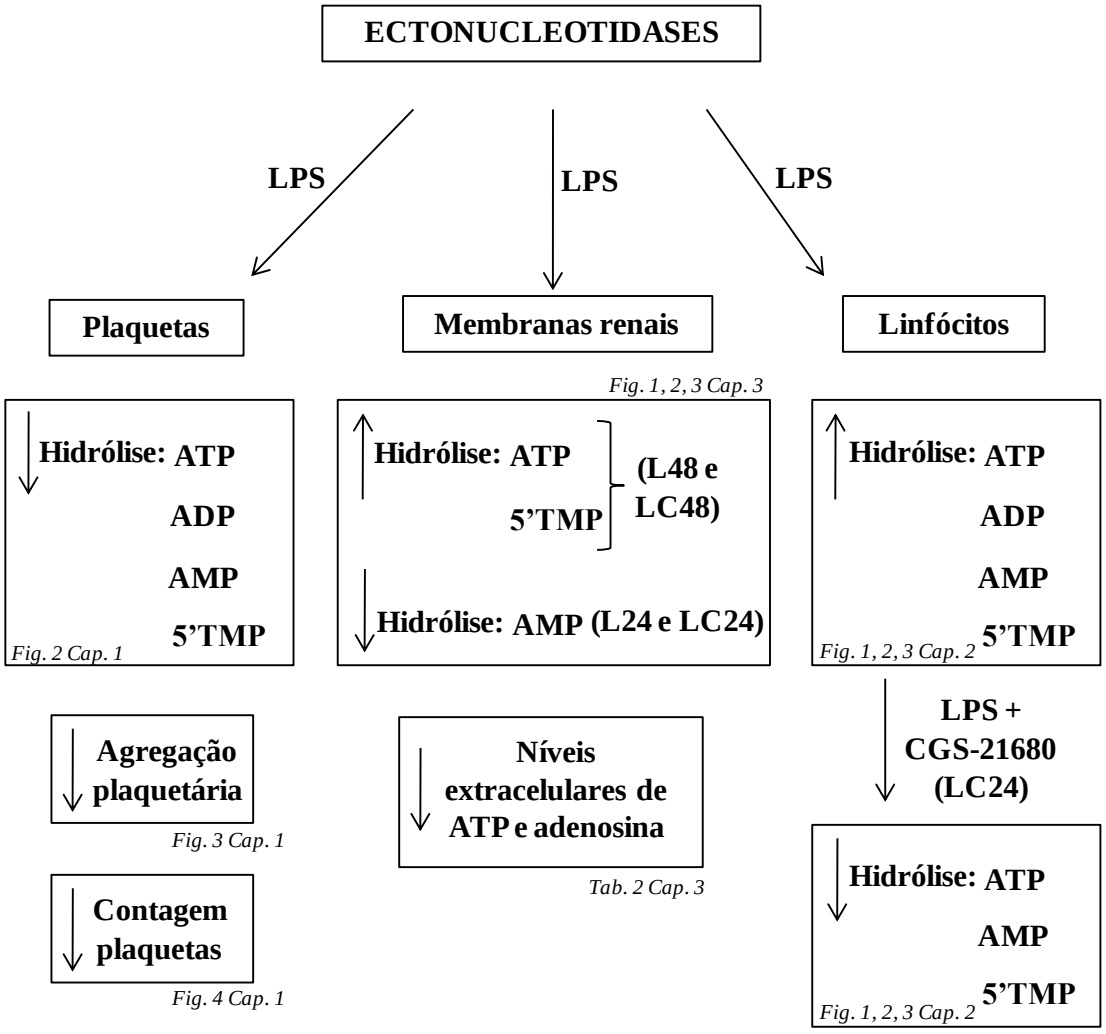

Figura 5: Representação esquemática das alterações promovidas pela endotoxemia (via injeção i.p. de LPS) e ativação dos receptores A2A (via administração i.p. de CGS-21680) em plaquetas de ratos, linfócitos de linfonodos mesentéricos de camundongos e preparações de membranas renais de camundongos.

4. Conclusões Gerais

Este estudo apresentou as seguintes conclusões:

I. Foi observada uma diminuição significativa sobre as hidrólises de ATP, ADP, AMP e 5'-TMP em plaquetas de ratos machos adultos após 48 horas de indução do modelo de endotoxemia. O LPS *in vitro* não promoveu alterações sobre as atividades ectonucleotidásicas em plaquetas de ratos;

II. Houve uma diminuição na agregação e contagem de plaquetas nos ratos que receberam injeção intraperitonial de LPS;

III. A indução do modelo de endotoxemia promoveu um aumento significativo na hidrólise de ATP, ADP, AMP e 5'-TMP em linfócitos de linfonodos mesentéricos de camundongos machos adultos. A co-administração de CGS-21680, agonista específico do receptor A_{2A} de adenosina foi capaz de prevenir esse aumento. Quando o CGS-21680 foi administrado 24 após o LPS, não foi capaz de reverter o aumento sobre as atividades ectonucleotidásicas promovido pela endotoxemia;

IV. A injeção de LPS e LPS+CGS promoveu um perfil de aumento sobre o padrão de expressão de transcritos de RNA mensageiro das enzimas Entpd3 e Enpp3 em linfonodos de camundongos. Para as outras enzimas não foram observadas alterações no padrão de expressão gênica das enzimas;

V. A indução do modelo de endotoxemia promoveu um aumento significativo na hidrólise de ATP e 5'TMP em membranas renais de camundongos machos adultos. A hidrólise do AMP foi diminuída significativamente 24 após a injeção de LPS. Após 48 horas de indução do modelo, a atividade de hidrólise de AMP volta aos níveis próximos do controle.

VI. Os transcritos de RNA mensageiro das enzimas Entpd3 e Enpp3, em rins de camundongos, apresentaram um aumento 48 horas após a injeção de LPS, e quando o CGS foi injetado 24 horas após o LPS esse aumento foi observado para os transcritos de Entpd3, Enpp1 e Enpp3;

VII. Foi observada uma diminuição nos níveis de ATP e adenosina extracelular, avaliados por cromatografia líquida de alta eficiência, nos grupos tratados quando comparados ao grupo controle, em preparações de membranas renais de camundongos machos adultos.

VIII. Destacamos que pela primeira vez foi demonstrada a interação cruzada entre a ativação dos receptores A2A de adenosina e as enzimas que modulam a produção do agonista desse receptor, o que pode fazer parte do complexo conjunto de ações que são desencadeadas pelo processo inflamatório.

5. Perspectivas

Este trabalho gerou uma série de perspectivas de estudos, visando uma melhor compreensão do papel dos receptores purinérgicos nos tipos celulares estudados, bem como a avaliação do sistema purinérgico após a indução do modelo de endotoxemia em outras células do sistema imune, tais como as células dendríticas. As seguintes perspectivas foram identificadas:

- Determinar o padrão de expressão gênica dos transcritos de RNA mensageiro dos receptores do tipo P1 de adenosina (A_1, A_{2A}, A_{2B} e A_3) em linfonodos mesentéricos e membranas renais de camundongos machos adultos após a indução do modelo de endotoxemia e a administração de CGS-21680;
- Detectar, através de análise por Western Blotting, se há alteração sobre o nível de expressão protéica dos receptores de adenosina em linfócitos e membranas renais de camundongos endotoxêmicos e que foram injetados com CGS-21680;
- Caracterizar a atividade das enzimas E-NTPDases, ecto-5'-nucleotidase e E-NPP em células dendríticas de camundongos;
- Determinar as atividades de hidrólise de ATP, ADP, AMP e 5'-TMP em células dendríticas de camundongos nos modelos de endotoxemia e após administração de CGS-21680;
- Avaliar o perfil de expressão gênica das ectonucleotidases e receptores de adenosina em células dendríticas após injeção de LPS e CGS-21680;
- Detectar, através de análise por Western Blotting, se há alteração sobre o nível de expressão protéica dos receptores de adenosina em células dendríticas de camundongos endotoxêmicos e que foram injetados com CGS-21680.

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