UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA PROFESSOR TUISKON DICK

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

INVESTIGAÇÃO DAS ECTONUCLEOTIDASES NA DIFERENCIAÇÃO DE MACRÓFAGOS E NA ATIVAÇÃO DE PLAQUETAS: O PAPEL DA HOMOCISTEÍNA

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica, como requisito parcial à obtenção do grau de Doutor.

Porto Alegre

2010

Para minha mãe, Goreti, e meu irmão, Francisco,

as pessoas mais gentis,

honradas e inteligentes

que conheço

"A vida é como andar de bicicleta. Para manter o equilíbrio, é preciso se manter em movimento."

Albert Einstein

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SUMÁRIO

LISTA DE ABREVIATURAS

ADP – adenosina difosfato (*Adenosine diphosphate*)

AMP – adenosina monofosfato (*Adenosine monophosphate*)

ATP - adenosina trifosfato *(Adenosine Triphosphate)*

DC – Células dendríticas *(dendritic cells)*

E-ATPases – ecto-ATPases

Ecto-5'-NT/CD73- ecto-5'-nucleotidase

E-NPPS – ecto- nucleotídeo pirofosfatase/fosfodiesterase *(Ectonucleotide pyrophosphatase/phosphodiesterase)*

E-NTPDase- ecto-nucleosídeo trifosfato difosfoidrolase

E-NTPDase1/CD39 - ecto-nucleosídeo trifosfato difosfoidrolase 1

ERK- MAPK regulada extracelularmente (*extracellular regulated MAPK*)

GPI- glicosil-fosfatidilinositol *(glicosil phosphatidylinositol)*

HUVEC - linhagem de células endoteliais de cordão umbilical humano *(human umbilical vein endothelial cells)*

ICAM-1 – molécula de adesão intracelular 1 *(Inter-Cellular Adhesion Molecule 1)*

IL-2 - interleucina-2 *(Interleukin-2)*

IL-6 - interleucina-6 *(Interleukin-6)*

IL-4- interleucina-4 *(Interleukin-4)*

IL-8/CXCL8- interleucina-8 *(Interleukin-8)*

IL-10- interleucina-10 *(Interleukin-10)*

IL-12- interleucina-12 *(Interleukin-12)*

IL-13- interleucina-13 *(Interleukin-13)*

IL-18- interleucina-18 *(Interleukin-18)*

IL-1β - interleucina-1 beta *(Interleukin-1beta)*

IL-1R - receptor de interleucina 1 *(Interleukin 1 receptor)*

INF-γ - interferon gama *(interferon gamma)*

iNOS – oxido nitrico sintetase induzivel *(inductible nitric oxide synthase)*

JAK- Janus cinase *(Janus-activated Kinase*)

LPS – lipopolisacarídeo *(Lipopolysccharide)*

MAPK - proteína cinase ativada por mitógenos (*Mitogen-Activated Protein Kinase*)

MIP – proteina inflamatória de macrófagos *(macrophage inflammatory protein)*

MMP-9- metalloproteinase de matriz 9 *(matrix metalloproteinase 9)*

NF-κB- fator nuclear kappa *(nuclear factor-kappaB)*

NK – matador natural *(Natural Killer)*

P1- receptor purinérgico metabotrópico para adenosina, dividido em quatro subtipos: A1, A2a, A2b e A3.

P2X- receptor purinérgico ionotrópico

P2Y- receptor purinérgico metabotrópico

PLA2 - fosfolipase A2 *(phospholipase A2)*

PLC- fosfolipase C *(phospholipase C)*

PLD - fosfolipase D *(phospholipase D)*

RhoA – família de genes homólogos a Ras, membro A *(ras homolog gene family, member A)*

ROS – espécies reativas de oxigênio *(reactive oxygen species)*

RRIP - receptores de reconhecimento intracelular padrão

PPP – plasma pobre em plaquetas *(plasma poor in platelet)*

PRP – plasma rico em plaqueta *(plasma rich in platelet)*

SAH – S-adenosilhomocisteína *(S-adenosylhomocysteine)*

SAM - *S*-adenosilmetionina *(S-adenosylmethionine)*

STAT6- membro da família de fatores de transcirção *(signal transducer and activator of transcription)*

TCR- receptor de célula T *(T cell receptor)*

TLR – receptores similares a *Toll (Toll-like receptors)*

TNF-α - fator de necrose tumoral- alfa *(Tumor Necrosis Factor alpha)*

TRAP- capacidade antioxidante total *(total radical-trapping antioxidant parameter)*

UDP – uridina difosfato *(uridine diphosphate)*

UTP - uridina trifosfato *(uridine triphosphate)*

VEGF - fator de crescimento vascular endotelial *(Vascular Endothelial Growth Factor)*

LISTA DE FIGURAS

LISTA DE TABELAS

RESUMO

Os nucleotídeos extracelulares modulam uma variedade de ações biológicas via ativação de receptores purinérgicos. Esses efeitos são controlados pela ação de ectonucleotidases, tais como as E-NTPDases e a ecto-5´-NT/CD73, as quais hidrolisam o ATP até adenosina no meio extracelular. Nas células imunes, o ATP pode atuar como uma molécula sinalizadora de perigo enquanto a adenosina, um produto da degradação do ATP, serve como um mecanismo que controla/limita a inflamação. Já, no sistema vascular, o ADP é um agonista fisiológico envolvido na hemostasia normal e na trombose. Considerando que os macrófagos são elementos chave para processos inflamatórios e quando estímulados exibibem um fenótipo pró-inflamatório/defesa (clássico/M1) ou antiinflamatório/reparatório (alterantivo/M2). O objetivo foi investigar a atividade e expressão das ectonuclotidases em diferentes fenótipos de macrófago e avaliar o efeito da homocisteína sobre essas enzimas em macrófagos e plaquetas.. As análises da diferenciação de macrófagos em fenótipo pró-inflamatório/M1 e antiinflamatório/M2 revelaram presença igual de receptores purinérgicos. Entretanto, mudança no perfil das ectonucleotidases como E-NTPDase1, E-NTPDase3 e ecto-5'-nucleotidase foram encontradas, sugerindo que os macrófagos devem alterar a casacata purinérgica durante a ativação fenotípica. No fenótipo pró-inflamatório/M1 houve uma diminuição na hidrólise de ATP, sugerindo um acúmulo do mesmo, enquanto no fenótipo antiinflamatório/M2 as enzimas conduzem para uma progressiva diminuição nas concentrações de nucleotídeos (ATP) e aumento na disponibilidade de adenosina. Já os macrófagos expostos a homocisteína apresentaram uma polarização para o fenótipo pro-inflamatório (M1) e nossos achados sugerem o envolvimento da E-NTPDase3 e da ecto-5'-nucleotidase em macrófagos nas complicações inflamatórias associadas a homocisteína. Nas plaquetas, as quais são elementos fundamentais no processo de trombogênese, a homocisteína causou uma diminuição na hifrólise de ADP. Essa elevação no nível de ADP ao redor das plaquetas devido a inativação das ectonucleotidases, causada pela homocisteína, deve estar contribuindo para o aumento do risco trombótico descrito em pacientes com hiperhomocisteinemia. Além disso, os animais que receberam homocisteína tiveram um aumento na agregação plaquetária induzida por ADP. Em conclusão, os resultados do presente estudo reforçam o envolvimento do sistema purinérgico em processos inflamatórios/trombóticos e apontam parar o desenvolvimeto de tratamentos para doenças inflamatórias/trobóticas.

ABSTRACT

Extracellular nucleotides modulate a variety of biological actions via purinergic receptor activation. These effects are modulated by ectonucleotidases, such as ENTPDases and ecto-5´-NT/CD73, which hydrolyze ATP to adenosine in the extracellular milieu. In the cells of the immune system, the ATP can act as danger signaling whereas adenosine, the ATP breakdown product, serves as a negative feedback mechanism to limit inflammation. Already, in the vasculare system, the ADP is a physiological agonist involved in normal hemostasis and thrombosis. Since, macrophages are key to inflammatory process, that depending on the microenvironmental stimulation exhibit pro-inflammatory/defense (classical/M1) and antiinflammatory/reparatory (alternative/M2) phenotype. The objective of this study was investigate the activity and expression of the ectonuclotidases in differential macrophage phenotype and evaluate the homocysteine (Hcy) effects on theses enzymes in macrophages and platelets. . The analysis of differential macrophages in phenotype pro-inflamatory/M1 and antiinflamatory/M2 showed the same expression to P1 and P2 purinoreceptors. However, change profile of the ectonucleotidases as E-NTPDase1, E-NTPDase3 and ecto-5'-nucleotidase enzymes in macrophages during phenotypic differentiation were found, suggesting that macrophages must alter the purinergic cascade during macrophages differentiation phenotypic. In the pro-inflamatory/M1 phenotype the ATP hydrolysis decreased, suggesting ATP accumulation. On the other hand, the antiinflamatory/M2 phenotype the enzymes lead to a progressive decrease in nucleotides (ATP) concentrations and an increase the adenosine availability. Already, the macrophages exposed to Hcy present a polarized pro-inflammatory profile (M1) and our findings suggest the involvement of the E-NTPDase3 and ecto-5'-nucleotidase in the inflammatory complications associates to homocysteine. In the Platelets, which are fundamental elements to the thrombogenesis process, the homocysteine decreased ADP hydrolysis. This elevation of ADP around of the platelets due inactivating of ectonucleotidase, probably by the indirect action of Hcy, may be contributing to increase thrombotic risk described in individuals with hyperhomocysteinemia. In addition, the animals that received Hcy treatment potentiate platelet aggregation induced by ADP. In conclusion, in the present study the results reinforce purinergic signaling involvement in inflammatory/thrombosis process and point to development of treatments to inflammatory/thrombotic diseases.

APRESENTAÇÃO

Esta tese está organizada em seções dispostas da seguite maneira: Introdução, Objetivos, Resultados, Discussão, Conclusões, Perspectivas, Referências Bibliográficas e Anexos.

A Introdução apresenta o embasamento teórico que nos levou a formular as propostas da tese, as quais estão determinadas na seção Objetivos.

A seção Resultados contém o artigo científico publicado e os trabalhos a serem submetidos, assim como os materiais, os métodos e as referências bibliográficas específicas de cada trabalho.

A seção Discussão contém uma interpretação geral dos resultados obtidos nos diferentes trabalhos.

A seção Conclusões aborda as conclusões gerais obtidas na tese.

A seção Perspectivas aborda as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos, dando continuidade a essa linha de pesquisa.

A seção Referências Bibliográficas lista as referências utilizadas na Introdução e Discussão da Tese.

A seção Anexos contém uma lista de outros trabalhos realizados em co-autoria durante todo o período de desenvolvimento do doutorado.

1. INTRODUÇÃO

1.1 Inflamação:

A inflamação é definida como uma resposta adaptativa desencadeada por estímulos e condições nocivas, tais como infecção, injúria e estresse tecidual ou mau funcionamento (Figura 1) (Majno, 2004; Medzhitov, 2008). Com isso, esse processo é fundamentalmente uma resposta protetora, cujo objetivo principal é livrar o organismo da causa inicial da lesão celular e das consequências dessa lesão. Portanto, tem como função destruir, diluir ou encerrar o agente lesivo, mas por outro lado, por em movimento uma série de eventos que cicatrizam e reconstituem o tecido danificado, ou seja, a resposta inflamatória está estritamente interligada ao processo de reparação (Serhan, 2007).

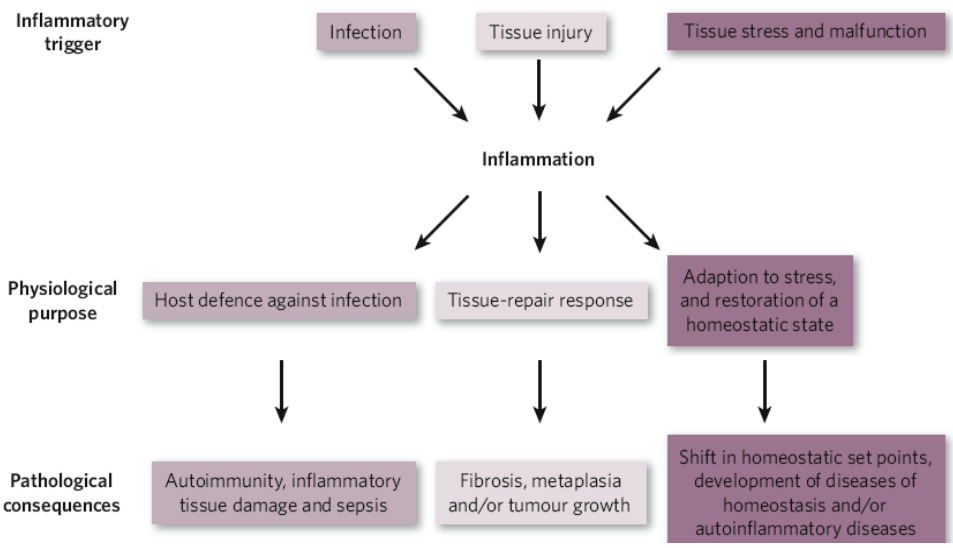

Figura 1. Causas de inflamação – conseqüências fisiológicas e patológicas (adaptado de Medzhitov, 2008).

Para que esse processo possa ser realizado de maneira adequada, uma série de elementos devem interagir, incluindo o plasma, células circulantes (monócitos, neutrófilos, eosinófilos, linfócitos, basófilos e plaquetas), vasos sanguíneos,

constituintes celulares e extracelulares do tecido conjuntivo (mastócitos, fibroblastos, macrófagos residentes e eventuais linfócitos) (Medzhitov *et al.*, 1997; Pober *et al*, 2007; Medzhitov, 2008). Essa interação resulta em uma série de ações que vão desde extravasamento e migração de células efetoras ao local da injúria, fagocitose, até a participação de mediadores químicos da inflamação, que incluem quimiocinas, citocinas, aminas vasoativas, eicosanóides e produtos da cascata proteolítica (Majno, 2004; Collins, 2000). Tais mediadores, atuando de maneira isolada, em combinação ou em sequência, amplificam a resposta inflamatória e influenciam na sua evolução. Além disso, as próprias células ou o tecido necrótico (danificado) também podem desencadear a elaboração de fatores químicos inflamatórios.

A inflamação é dividida em padrões agudo e crônico (Collins, 2000; Majno, 2004). A inflamação aguda é desencadeada por uma infecção ou injúria tecidual e tem uma duração relativamente curta, de minutos, algumas horas ou alguns dias. O reconhecimento inicial é feito por macrófagos residentes e mastócitos, os quais geram a produção de uma variedade de mediadores inflamatórios que resultam nas principais características da inflamação aguda: exsudação de líquidos e proteínas plasmáticas (edema) e a migração de leucócitos, predominantemente do tipo neutrófilo. Então, uma resposta inflamatória aguda bem sucedida resulta na eliminação dos agentes infecciosos, seguido por uma fase de resolução e reparação, a qual é mediada principalmente por macrófagos residentes e recrutados.

Se a resposta à inflamação aguda falha na tentativa de eliminar o agente causador do dano, o processo inflamatório persistente adquire novas características e, dessa forma, pode tornar-se um processo inflamatório crônico. A inflamação crônica tem uma duração mais longa (semanas, meses) e está associada principalmente à presença de linfócitos e macrófagos e caracteriza-se por apresentar inflamação ativa,

destruição tecidual e tentativa de reparação do tecido (Hotamisligil, 2006). Embora possa suceder à inflamação aguda, a inflamação crônica com frequência começa de maneira insidiosa, com uma resposta de baixo grau, latente e muitas vezes assintomática. A inflamação crônica pode originar-se a partir de infecções persistentes, auto-imunidade e exposição a agentes potencialmente tóxicos, podendo ser de origem exógena ou endógena (como por exemplo, o mau funcionamento dos tecidos) (Majno, 2004; Hotamisligil, 2006; Collins, 2000).

Por fim, muitos são os fatores que podem modificar o curso da inflamação. Portanto, uma resposta inflamatória efetiva normalmente envolve um fino ajuste entre mediadores pró- e anti-inflamatórios. Sendo assim, o menor desajuste dessa interação pode acarretar em prejuízos aos tecidos, propiciando o desenvolvimento e/ou agravamento de processos patológicos.

1.2 Macrófagos:

Os macrófagos juntamente com os neutrófilos constituem uma das primeiras linhas de defesa contra infecções após as barreiras naturais da pele e mucosas. Dessa forma, contribuem para o reconhecimento, captação, destruição de microorganismos e parasitas multicelulares, na apresentação de antígenos (células apresentadoras de antígenos) para linfócitos T e B e durante os processos de inflamação aguda e crônica (Hoebe *et al.*, 2004; Nathan, 2008). Os macrófagos são capazes de detectar os "sinais endógenos de perigo" presentes em restos celulares e em células necróticas, através da sensibilização dos receptores do tipo *Toll-like* (TLRs) (Park *et al.*, 2004; Rakoff *et al.*, 2004; Chen *et al.*, 2004; Kono *et al.*, 2008), dos receptores de reconhecimento intracelular padrão (RRIP) e do receptor de interleucina 1 (IL-1R) (Bianchi, 2007; Rock *et al.*, 2008).

Os macrófagos estão presentes virtualmente em todos os tecidos e se diferenciam a partir de células periféricas mononucleares (monócitos), os quais migram para os tecidos após sua passagem transitória na circulação ou em resposta à inflamação (Gordon *et al.,* 2005). Como estão localizados em regiões variadas do organismo, os macrófagos adquirem morfologia e propriedades funcionais que são características de cada tecido onde residem. Como exemplo, podemos citar as células de Kupffer, no fígado, e as células da microglia, no cérebro (Gordon, 2003).

Os macrófagos habitualmente se mantêm em um caráter quiescente e nessa condição eles não liberam citocinas. Porém, esse tipo celular apresenta uma extraordinária plasticidade, o que lhe permite responder eficientemente aos sinais do ambiente,mudando de fenótipo (Gordon, 2003). Sendo assim, na tentativa de seguir a nomenclatura utilizada pela literatura para caracterizar a resposta imunológica das células T, onde existe a resposta Th1 (pró-inflamatória) e a resposta Th2 (anti-inflamatória) (Martinez *et al.,* 2008), os macrófagos têm sido classificados de acordo com o seu fenótipo em M1 e M2, os quais representam os extremos de ativação (Figura 2A) (Gordon, 2003; Mosser, 2003;2008; Mantovani *et al.,* 2004; Martinez *et al.,* 2008). Nessa classificação a designação M1 foi reservada para macrófagos ativados classicamente e a designação M2 para macrófagos ativados alternativamente (Gordon, 2003; Mosser, 2003;2008;; Mantovani *et al.,* 2004). Entretanto, há discussões quanto à forma dessa classificação. Outros autores sugerem que a classificação dos macrófagos deve ser baseada na função que eles exercem na manutenção da homeostasia (Figura 2B) (Mosser *et al.,* 2008). Nessa proposta, três seriam as funções: macrófagos classicamente ativados (equivalente à ativação clássica), macrófagos de regeneração (equivalente à ativação alternativa) e macrófagos regulatórios. No entanto, como não há

nenhum consenso até o momento na literatura, a classificação M1 (ativação clássica) e M2 (ativação alternativa) continua prevalecendo e será utilizada neste trabalho.

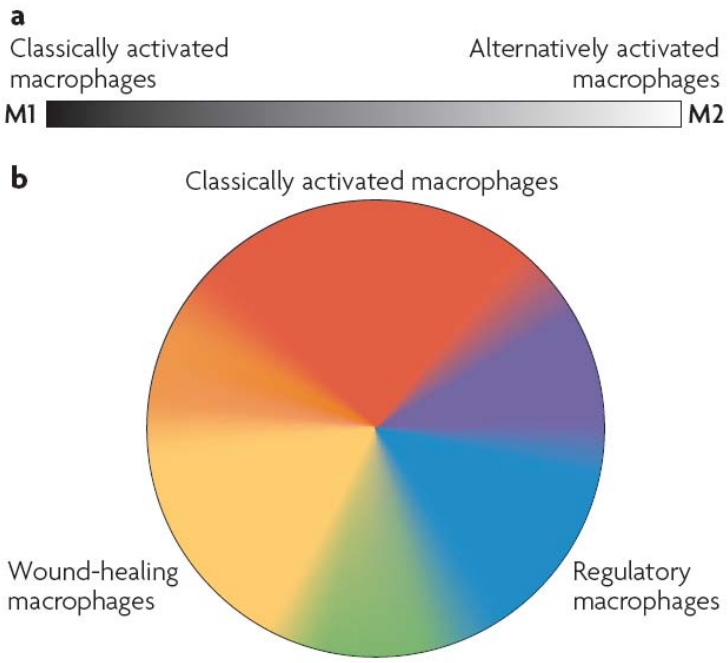

Figura 2. Ativação de macrófagos. (A) Nomenclatura em uma escala linear de duas designações de macrófagos. (B) Três populações de macrófagos dispostos de acordo com as 3 cores primárias (adaptado de Mosser *et al.*, 2008).

A ativação clássica é desenvolvida em resposta ao interferon-gama (INF-γ), citocina produzida durante a resposta Th1, também produzida por células *natural killer* (NK) em conjunto com agonistas de TLRs (*toll-like receptors*), tais como lipopolissacarídeo (LPS) (Gordon, 2007; Mantovani *et al.*, 2004; O'Shea *et al.*, 2008). Esses ligantes induzem a diferenciação clássica dos macrófagos via a ativação de fatores de transcrição que incluem principalmente o Fator nuclear kappa B (NF-kB) e as proteínas cinaseses ativadas por mitógeno (MAPKs) (O'Shea *et al.*, 2008). Esse tipo de ativação caracteriza-se pela secreção de efetores pró-inflamatórios, os quais geram o processo inflamatório necessário para a remoção e degradação do agente causal. Essa ativação deve ser rigidamente controlada, pois as citocinas e mediadores (TNF-α, IL-6, IL-12, IL-8, entre outras) que essa resposta pró-inflamatória produz podem causar dano

às células adjacentes ao foco da inflamação (MacMicking *el al.,* 1997; Sunderkotter *et al.,* 2004; Strauss-Ayali et al., 2007).

Por outro lado, a ativação alternativa é desenvolvida em reposta principalmente à exposição à IL-4 (Stein *et al.,* 1992, Raes *et al.,* 2002; Gordon, 2003, 2007) e/ou IL-13 (Brubaker *et al.,* 2000; Raes *et al.,* 2002; Gordon, 2003, 2007) que são citocinas produzidas durante a resposta Th2, as quais são em sua maioria sintetizadas por uma subpopulação de células T-$CD4^{+}$. As citocinas IL-4 e IL-13 atuam através do receptor de cadeia comum IL-4-Rα via ativação intracelular, que envolve membros da JAK (*Janus-activated cinase*) e da família de ativador de transcrição STAT6 (Nelms *et al.,* 1999; Varin *et al.,* 2009). A ativação alternativa caracteriza-se pela expressão de um repertório molecular que leva à tolerância e à resolução da inflamação (Gratchev *et al.,* 2005). Essa via proporciona uma diminuição do processo inflamatório, angiogênese, eliminação de restos celulares e corpos apoptóticos, além de contribuir para a deposição de matriz extracelular (Goerdt *et al.,* 1999; Gratchev *et al.,* 2001; Mosser, 2003). Com isso, os macrófagos são células chave na iniciação, propagação e resolução da inflamação (Porcheray *et al.,* 2005).

1.3 Plaquetas:

As plaquetas apresentam-se como células incompletas formadas por porções do citoplasma das células que lhes deram origem - os megacariócitos (Lee *et al.,* 1998; Lorenzi, 2003). Segundo Lee, a composição química de uma plaqueta é de difícil determinação, pois a mesma absorve uma variedade de substâncias do plasma (Lee *et al.,* 1998). O peso seco de uma plaqueta demonstra que ela é composta por 50% de proteínas, e o restante de carboidratos, lipídios, minerais como magnésio, cálcio,

potássio, zinco, etc. (Lee *et al.,* 1998). Conforme mostrado por vários autores, (Biggs, 1975; Bakker et al., 1994; Lorenzi, 2003) a morfologia e a composição química plaquetária podem ser caracterizadas como: 1) presença de uma porção mais externa, onde encontram-se antígenos, glicoproteínas e vários tipos de enzimas, os quais são responsáveis pela interação entre plaquetas e endotélio vascular; 2) presença de uma porção mais interna onde localiza-se a membrana plaquetária; quase a totalidade das proteínas da membrana são glicoproteinas denominadas como GPI, II, III, IV, algumas apresentam função de receptores específicos para determinados fatores da coagulação, tais como GPIb, receptor para trombina e fator de Von Willebrand; o complexo GPIIb-IIIa e receptor para o fibrinogênio; 3) no citosol estão os microtúbulos que são aparelhos de contração formados por tubulina, que se conectam com microfilamentos formados pela actina e juntos orientam os movimentos plaquetários para a eliminação de produtos secretados e para a retração do coágulo; 4) na zona de organelas encontram-se várias estruturas entre elas os grânulos alfa que contem proteínas como o fator plaquetário 4, a beta-tromboglobulina, o fator de crescimento derivado de plaquetas, os fatores de coagulação (V, VIII - reagente) e a albumina. Também há a presença dos corpos densos que contêm cálcio, serotonina, pirofosfato, antiplasmina, ADP e ATP plaquetários (65%). Além disso, há a presença de lisossomas e de mitocôndrias (Biggs, 1975).

O primeiro sinal de ativação plaquetária é sentido na membrana externa onde fatores agonistas se ligam aos seus receptores específicos, com propagação do fluxo de íons Ca^{2+} do exterior para o interior da célula (Bakker *et al.,* 1994; Lorenzi, 2003;). Assim que um agonista como a trombina ou o colágeno liga-se à membrana celular, a agregação plaquetária é estimulada, ocorrendo a fase de secreção, sendo liberadas substâncias contidas nos grânulos densos (ADP, cálcio, serotonina), catepsinas, enzimas, formação de tromboxano A2 e fator plaquetário 3, um fosfolipídeo de

membrana que promove a ativação de fatores plasmáticos da coagulação (Birck *et al.*, 2002; Lorenzi, 2003).

1.4 Sistema Purinérgico:

1.4.1 Nucleotídeos Extracelulares

O nucleotídeo adenosina-5´-trifosfato (ATP) está normalmente presente em todas as células vivas e é reconhecido pelo seu papel intracelular no metabolismo energético (Agteresch *et al.,* 1999). O primeiro relato envolvendo as ações extracelulares de nucleotídeos e nucleosídeos purínicos foram descritas em 1929 por Drury and Szent-Gyorgyi em sistema cardiovascular. Desde então, os estudos sobre os efeitos dos nucleotídeos extracelulares, em especial o ATP, continuaram avançando e uma série de outras ações foram observadas em diferentes processos biológicos, incluindo neurotransmissão, contração muscular, vasodilatação, metabolismo ósseo, metabolismo do glicogênio hepático, agregação plaquetária, inflamação entre outros (Agteresch *et al.,* 1999; Hoebertz *et al.,* 2003; Burnstock & Knight, 2004; Bours *et al.*, 2006). Assim, após décadas de estudos, ficou claro que o ATP e seus produtos de degradação ADP, AMP e adenosina, assim como outros nucleotídeos e nucleosídeos (UTP, UDP), fazem parte de um conjunto de moléculas bem estabelecidas como mensageiros extracelulares, que exibem uma variedade de efeitos sobre os mais diversos tecidos e sistemas (Dombrowski *et al.*, 1998; Bours *et al.,* 2006).

Os nucleotídeos encontram-se presentes em altas concentrações no citoplasma celular (ATP de 3-10 mM;UTP de 0,5-1 mM), enquanto que no compartimento extracelular seus níveissão mantidos na ordem de nanomolar (ATP 1-10 nM) (Di Virgilio, 2005). Concentrações aumentadas de nucleotídeos podem ser liberadas no

meio extracelular em resposta a diferentes estímulos ou condições, tais como lise celular, hipóxia e inflamação (Lazaroswski et al, 1997). Além destas formas de liberação relacionadas principalmente ao dano, atualmente é reconhecido que o ATP liberado de células intactas é um mecanismo fisiológico (Bodin *et al.*, 2001; Lazarowski *et al.*, 2003; Schwiebert *et al.*, 2003; Dubyak, 2006). Até o momento, não é claro se os nucleotídeos (ATP, ADP, UTP e UDP) são liberados por mecanismos comuns (Abbracchio *et al.*, 2008). Por exemplo, estudos recentes comprovam que o ATP é liberado por exocitose vesicular nas células neuronais (Pankratov *et al.*, 2007; Bowser *et al.*, 2007), porém para o ATP liberado de células não neuronais há uma extensa discussão sobre o exato mecanismo envolvido na sua liberação e vários mecanismos de transporte têm sido sugeridos, os quais incluem transportadores *ATP-binding cassette* (ABC), conexina, panexina ou liberação vesicular (Robitaille, 1998; Bodin *et al.*, 2001; Lazarowski *et al.*, 2003; Stenberg et al., 2003; Schwiebert *et al.*, 2003; Dahl *et al.*, 2006; De Vuyst *et a.*, 2006). Uma vez liberados no meio extracelular, os nucleotídeos interagem com receptores purinérgicos específicos, mediando eventos na resposta imune, inflamação, agregação plaquetária, entre outros (Burnstock, 2006; 2007). Essa sinalização é finalizada pela ação de ectoenzimas que hidrolisam os nucleotídeos até os seus respectivos nucleosídeos no meio extracelular (Zimmerman, 1994).

O sistema de sinalização purinérgica atua de forma integrada com outras células imunes no controle do processo inflamatório. Os nucleotídeos, principalmente o ATP, liberados para o espaço extracelular, rapidamente alertam o sistema imune para dano/injúria celular que pode ser de origem endógena ou exógena (Bours *et al.*, 2006). Estudos revelam que o ATP está envolvido em diversas funções do sistema imune: nas células T, o ATP é importante na secreção de INT-γ e IL-2, importantes mediadores na ativação da resposta imune (Langston *et al.*, 2003); o ATP está envolvido no

recrutamento de monócitos circulates para tecidos alvo (Ventura *et al.,* 1991;1995); nas células dendríticas (DC) o ATP induz migração e diferenciação, que pode alterar a resposta imune (la Sala *et al.,* 2003); e nos macrófagos estimula a produção de IL-1α (Hogquist *et al.,* 1991; Hamon *et al.,* 1997; Lemaire *et al.,* 2003), IL-1β (Donnelly-Roberts *et al.,* 2004; Elssner *et al.,* 2004), IL-18 (Mehta *et al.,* 2001; Muhl *et al.,* 2003) e TNF-α (Into *et al.,* 2002a; Guerra *et al.,* 2003). Além da produção de mediadores solúveis de inflamação pelos macrófagos, os nucleotídeos como ATP, UTP e mais recentemente o UDP estão envolvidos no processo de fagocitose e na síntese de IL-6 (Ichinose, 1995; Koizumi *et al.,* 2007; Bar *et al.,* 2008).

O ADP é amplamente reconhecido como agente pró-agregante. A estimulação dos receptores purinérgicos expressos nas plaquetas, células endoteliais e leucócitos resulta em ativação dessas células que culmina na formação do trombo vascular e da inflamação (Robson *et al.,* 2001). As ações do AMP no processo inflamatório e nos demais tecidos são menos definidas.

A adenosina, produto de degradação doATP, também é considerada uma molécula sinalizadora de dano celular, exercendo em geral ações contrárias as do ATP extracelular (Frantz *et al.,* 2005; Bours *et al.,* 2006). A adenosina age mediando uma resposta imunossupressora para proteger os tecidos adjacentes à inflamação dos ataques promovidos pelas células de defesa (Sitkovsky *et al.,* 2005). Essas ações imunes envolvendo a adenosina têm sido caracterizadas em diversos estudos: em neutrófilos ativados, a adenosina inibe a produção do fator de ativação plaquetária (PAF), além de inibir a produção de proteínas inflamatórias de macrófagos (MIPs), que estão envolvidas na migração dos neutrófilos para o foco inflamatório (Stewart *et al.,* 1993); em macrófagos, a adenosina causa a diminuição da produção de IL-12, uma potente citocina pró-inflamatória, e de INF-γ, molécula central na ativação de

monócitos/macrófagos (Haskó *et al.,* 1998). Além disso, a adenosina está envolvida no aumento da síntese e expressão do fator de crescimento do endotélio vascular (VEGF), importante na angiogênese e na permeabilidade vascular; em linfócitos a adenosina parece inibir a ativação dos receptores de célula T (TCRs), essenciais para a resposta imune, além de diminuir a migração de linfócitos para o foco da inflamação (Odashima *et al.*, 2005)

1.4.2 Receptores Purinérgicos

Os efeitos biológicos dos nucleotídeos e nucleosídeos são exercidos através da sensibilização de distintos purinoreceptores, denominados de P1 e P2, os quais foram originariamente identificados por Burnstock e colaboradores (Burnstock, 1976, 1978). A heterogeneidade de respostas biológicas mediadas pelos receptores purinérgicos depende principalmente da distribuição destes na membrana das células, assim como a disponibilidade de seus agonistas, os quais são produzidos por ação de ectoenzimas localizadas na membrana plasmática e que catalisam a hidrólise sequencial dos nucleotídeos até os seus respectivos nucleosídeos no meio extracelular. Como consequência, o número de funções mediadas por esses receptores é amplo e varia desde proliferação e diferenciação celular, neurotransmissão até fenômenos mais complexos como fertilização, angiogênese e resposta imune (Burnstock, 2006). Até o momento foram clonados e caracterizados 15 subtipos de receptores P2 (Burnstock, 2004) e 4 subtipos de receptores P1 (Fredholm *et al.*, 2001).

Os receptores metabotrópicos P1 para adenosina são divididos em quatro subtipos A_1, A_{2A}, A_{2B} e A_3 (Abbracchio *et al,* 1994), podendo ser identificados pela distinta afinidade de ligação a agonistas e antagonistas e pela ativação de vias

sinalizadoras acopladas à proteína-G (Palmer and Stiles, 1995). Dependendo do tipo celular, outras combinações de proteína-G podem atuar, mas uma característica comum é que todos os receptores adenosinérgicos (P1) podem ativar pelo menos uma das subfamílias das MAPK (Dare *et al.,* 2007; Abbracchio *et al.,* 2009).

Os receptores P2 são subdivididos em P2X e P2Y. Os receptores do tipo P2X (1–7) estão ligados a canais iônicos, enquanto os receptores P2Y (1, 2, 4, 6, 11–14) estão acoplados à proteína-G e podem ser sensibilizados por diferentes nucleotídeos (Di Virgilio *et al.,* 2001; Robson *et al.,* 2006). A figura 3 ilustra a estrutura dos receptores P2X, P2Y e P1..

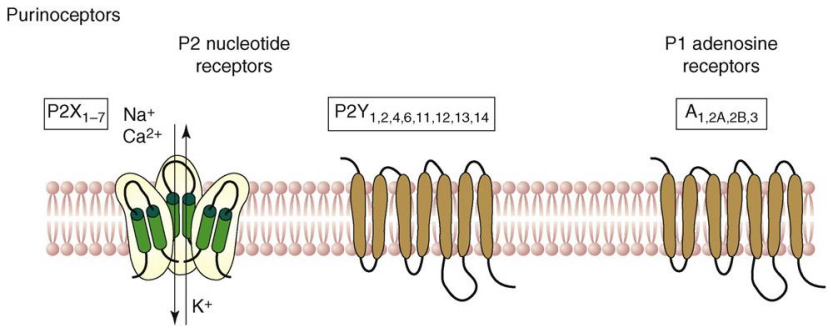

Figura 3. Estrutura dos receptores P1, P2Y e P2X (Adaptado de Abbracchio *et al.,* 2009).

Os receptores P2Y são compostos por 7 domínios transmembrana acoplados à proteína G. Os receptores $P2Y_1$, $P2Y_6$, e $P2Y_{12}$ são ativados principalmente por nucleosídeos difosfatos, enquanto $P2Y_2$ e $P2Y_4$ são ativados principalmente por nucleosídeos trifosfatos (Sévigny *et al.,* 2002; Burnstock, 2007). Os receptores $P2Y_2$, $P2Y_4$ e $P2Y_6$ são ativados por ambos nucleotídeos púricos e pirimídicos, já os receptores $P2Y_1$, $P2Y_{11}$, e $P2Y_{12}$ são ativados somente por nucleotídeos púricos. Quanto à ativação, os receptores P2Y podem ser divididos em 2 subgrupos: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ e $P2Y_{11,}$ os quais são acoplados principalmente G_q/G_{11} e ativam fosfolipase C/IP3 via de liberação de Ca^{2+} do retículo endoplasmático (Abbracchio *et al.,* 2006;

Burnstock, 2007). Já o outro subgrupo é composto pelos receptores $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$, os quais usam quase que exclusivamente $G_{i/o}$, o qual inibe a adenilato ciclase e modula canais iônicos (Burnstock, 2004). A ativação dos receptors P2Y, também está associada à estimulação das vias fosofolipase A_2, fosfolipase D das MAPKs, Rho-dependente de cinase e tirosina cinase (White *et al.*, 2003; Burnstock *et al.*, 2004; Burnstock, 2007; Abbracchio *et al.*, 2009).

Os receptores P2X desencadeiam seus efeitos via abertura de um canal iônico na membrana celular permeáveis a Na^+, K^+ e Ca^{+2} (Abbracchio *et al.*, 1994). O ATP é praticamente o único agonista. Os receptores P2X ocorrem normalmente como trímeros estáveis e todos eles podem ser desensibilizados, porém em escalas de tempo diferenciadas: desensibilização rápida ($P2X_{1,3}$) e desensibilização lenta ($P2X_{2,4\text{-}7}$) (North, 2002; Burnstock *et al.*, 2004; Burnstock, 2007). O receptor $P2X_7$ é um membro diferenciado dentro da família dos P2X. Como os outros componentes, o $P2X_7$ é receptor de canal iônico, porém esse receptor difere dos outros membros em vários e importantes aspectos: o domínio C terminal desse receptor possui 200 aminoácidos a mais do que os outros membros, uma característica que tem sido hipotetizada por conferir propriedades não usuais a esse receptor (North, 2002; Egan *et al.*, 2006; Garcia-Marcos *et al.*, 2006). O receptor $P2X_7$ pode funcionar também como um receptor bifuncional: como um canal não seletivo de cátions ou como um grande poro não seletivo com permeabilidade para moléculas com massa molecular de ~900 Da (North, 2002). Essa bifuncionalidade depende da expressão do receptor, da duração e intensidade do estímulo. Além disso, o receptor $P2X_7$ é o único entre a família dos receptores P2X que é capaz de estimular a liberação de interleucina-1β, assim como algumas outras citocinas pró-inflamatórias (Ferrari *et al.*, 1996;1997b; Kahlenberg & Dubyak, 2004; Sluyter *et al.*, 2004b).

1.4.3 Ectonucleotidases

Um importante fator que modula a resposta mediada por nucleotídeos nos seus respectivos receptores, é o metabolismo extracelular dos mesmos, catalisado pelas ectonucleotidases. Estas enzimas incluem: as ecto-nucleosídeo-trifosfo-difosfoidrolases (E-NTPDases), as ecto-nucleotídeo pirofosfatase/fosfodiesterase (E-NPPs), as ecto-fosfatases alcalinas e a ecto-5'-nucleotidase/CD73 (5'-NT/CD73), as quais têm sido detalhadamente estudadas nos últimos anos (Zimmermann, 2001; Robson *et al.*, 2006) (Figura 4).

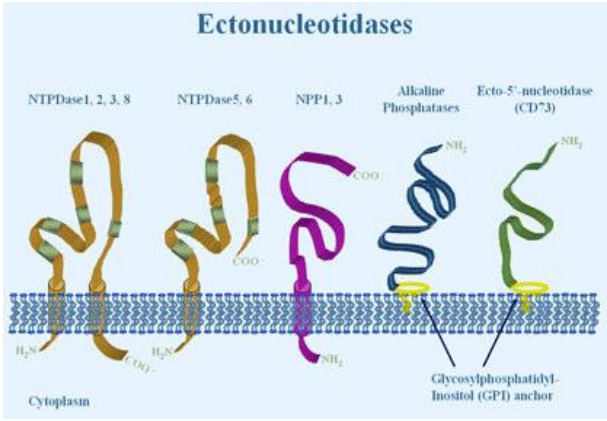

Figura 4. Topografia de membrana das diferentes famílias de enzimas que compõem o grupo das ectonucleotidases (adaptado de www.crri.ca/sevigny.html).

Através de reações sucessivas, essas enzimas constituem uma cascata enzimática altamente eficiente, hábil em controlar a concentração e o tempo em que essas moléculas sinalizadoras permanecem no espaço extracelular (Zimmermann, 2001).

1.4.3.1 NTPDases

As NTPDases, antigamente denominadas em conjunto como ecto-apirases, ou E-ATPases, é a família das ectonucleotidases mais numerosa e também a mais

predominante entre os tecidos (Plesner, 1995; Zimmermann, 2000). Essas enzimas hidrolisam nucleosídeos tri- e difosfatados até seus respectivos nucleosídeos monofosfatados e sua ativadade catalítica é dependente de cátions divalentes tais como: Ca^{2+} e Mg^{2+} (Bigonnesse *et al.,* 2004; Lavoie *et al.,* 2004; Vorhoff *et al.,* 2005). A família das NTPDases é composta por 8 membros. Dentro dessa família, as NTPDases1, 2, 3 e 8 são as de maior destaque no que diz respeito ao controle da resposta purinérgica, visto que possuem seu sítio de ação, na membrana plasmática, voltado para o espaço extracelular (ecto-enzimas) (Zimmemann 2000, 2001; Robson *et al., 2005,* 2006). As NTPDases5 e 6 são encontradas intracelularmente, podendo sofrer secreção após expressão heteróloga (Zimmemann 2000, 2001; Robson *et al., 2005,* 2006). As NTPDases4 e 7 são localizadas intracelularmente com seus sítio ativos voltadas para o lúmen de organelas citoplasmáticas, sendo portanto também consideradas ecto-enzimas.

Todos os membros desta família apresentam em comum na sua estrutura, a qual confere ao grupo uma identidade, cinco domínios chamados regiões conservadas de apirase (ACR), que estão diretamente envolvidos na atividade catalítica da enzima e/ou na integridade estrutural das mesmas (Handa *et al.,* 1996; Vasconcelos *et al.,* 1996).

As 4 formas encontradas na superfície celular, NTPDase1, 2, 3 e 8 , podem ser diferenciadas entre si de acordo com a velocidade de hidrólise do substrato e consequentemente na formação do produto, o que é de extrema relevância, pois reflete diretamente na sinalização desencadeada pelos nucleotídeos (Figura 5). A NTPDase1 hidrolisa na mesma proporção ATP e ADP, enquanto a NTPDase2 tem uma alta preferência por ATP (hidrolisa cerca de 30 vezes mais o ATP que o ADP) e anteriormente, era classificada como uma ecto-ATPase. Já a NTPDase3 e a NTPDase8 revelam uma preferência intermediária por ATP e ADP (Zimmermann, 2001; Kukulski *et al.,* 2005). Presumivelmente, diferenças na sequência dos aminoácidos, assim como

na estrutura secundária, terciária e quaternária devem influenciar as diferentes propriedades catalíticas das NTPDases (Robson *et al.*, 2006).

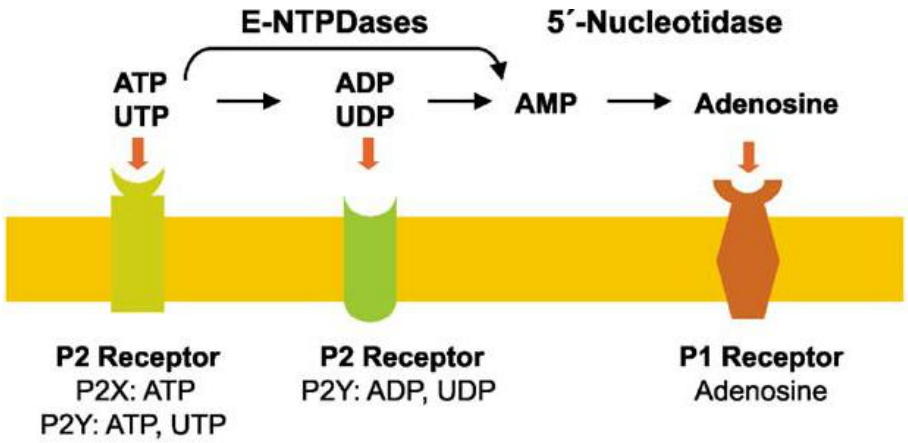

Figura 5. Metabolismo extracelular de nucleotídeos pela ação conjunta das NTPDases e Ecto-5´-nucleotidase e o controle de ativação de receptores purinérgicos P1 e P2 (Adaptado de Robson et al., 2006).

No contexto de funcionalidade, essas enzimas têm papel crucial no controle e na disponibilidade de nucleotídeos extracelulares para os receptores purinérgicos. Os primeiros e mais relevantes estudos das ações biológicas das NTPDases estão relacionados ao sistema cardiovascular e ao sistema nervoso porém, nos últimos anos, outros sistemas vêm ganhando espaço, com destaque para o sistema imune.

A primeira caracterização molecular de um membro da família das NTPDases foi a NTPDase1 originalmente classificada como uma ATP-difosfoidrolase e assim nomeada na literatura até meados dos anos 80 (EC 3.6.1.5; apirase). Experimentos com ATP-difosfoidrolase (apirase) solúvel purificada de batata (Handa and Guidotti, 1996), e com a enzima proveniente de diferentes tecidos de mamíferos (Kaczmarek *et al.*, 1996) confirmaram a homologia e comprovaram que essa enzima é o antígeno de ativação celular CD39, um marcador de células B (Maliszewski *et al.*, 1994). Estudos posteriores demonstraram que a NTPDase1 é expressa por células endoteliais, células musculares lisas vasculares e células do sistema imune, entre outros (Robson *et al.*,

2006). Por sua habilidade em hidrolisar o ATP quase que diretamente até AMP, ela pode bloquear a agregação plaquetária por prevenir a ação do ADP que é um forte um ativador plaquetário (via $P2Y_1$ e $P2Y_{12}$), auxiliando no fluxo sanguíneo (Frassetto *et al.*, 1993; Pilla *et al*., 1996; Kaczmarek *et al.*, 1999; Pinsky *et al.,* 2002). A geração de um camundongo nocaute para a NTPDase1 veio corroborar com a sua importância no controle da sinalização purinérgica em processos hemostáticos e de trombo regulação (Enjyoji *et al*., 1999). A NTPDase1 também pode reduzir ou diluir os efeitos do ATP e UTP sobre receptores como $P2X_{1\text{-}7}$ e $P2Y_{2,4,11}$ (Kukulski et al., 2005). Fato comprovado por estudos que mostram seu envolvimento na regulação da migração de leucócitos para tecidos inflamados, pela modulação na expressão da glicoproteína de adesão $\alpha_M\beta2$-integrina na superfície dessas células, via estimulação de $P2X_7$ (Hyman *et al.,* 2009). Outro estudo confirmou o papel chave da NTPDase1, em macrófagos, no controle da secreção de IL-1β e IL-18, via ativação do $P2X_7$ (Lévesque *et al.*, 2010).

A NTPDase2 está associada à camada adventícia do endotélio vascular (Robson *et al*., 2005); a astrócitos em cultura (Wink *et al.*, 2006); a células de Schwann e a outras células gliais do sistema nervoso central e periférico (Langer *et al.*, 2007). Essa enzima está envolvida na diferenciação de células tronco no sistema nervoso (Mishra *et al, 2006;* Zimmermann, 2006) e foi recentemente demonstrado estar envolvida na iniciação do desenvolvimento dos olhos (Massé *et al*., 2007). Por sua característica em catabolizar mais os nucleotídeos trifosfatados, é previsto que reduza ou termine com a ativação dos receptores $P2X_{1\text{-}7}$ e $P2Y_{2,4,11}$ mas, em contra partida, promove a formação de ligantes para receptores estimulados por nucleosídeos difosfatados $P2Y_{1,6,12,13}$. Essa potencial função foi corroborada por um ensaio de agregação plaquetária, no qual a sua atividade facilitou a agregação pela geração de ADP na presença de ATP (Sévigny *et al.*, 2002)

As NTPDase3 e 8, as quais são expressas no cérebro e nos canalículos biliares, respectivamente, mostram padrões intermediários de formação de produtos (Kukulski *et al.*, 2005; Robson *et al.*, 2006). Pelo seu perfil de hidrólise, elas podem atenuar respostas de nucleotídeos trifosfatados a seus receptores ($P2X_{1\text{-}7}$ e $P2Y_{2,4,11}$) e favorecer a ligação de nucleosídeos difosfatados aos seus receptores ($P2Y_{1,6,12,13}$). A relevância fisiológica dessas duas enzimas tem sido menos estabelecida quando comparada com a NTPDase1 e 2. A tabela 1 apresenta os membros da família das NTPDases e as respectivas preferências por substrato.

NOMENCLATURA ATUAL	NOMENCLATURA ANTIGA	PREFERÊNCIA POR SUBSTRATO
NTPDase 1	CD39, ecto-ATP difosfoidrolase, ecto-apirase, ecto-ATPDase	ATP=ADP (1:1)*
NTPDase 2	CD39L1, ecto-ATPase	ATP >>>>ADP (30:1)*
NTPDase 3	CD39L3, HB6	ATP>ADP (3:1)*
NTPDase 4	UDPase (hLALP70v), hLALP70	UDP>GDP,CDP
NTPDase 5	CD39L4, ER-UDPase	UDP>GDP,IDP>>>ADP, CDP
NTPDase 6	CD39L2	GDP>IDP>>UDP,CDP>>ADP
NTPDase 7	LALP1	UTP, GTP, CTP
NTPDase 8	-	ATP>ADP (2:1)*

Tabela 1. Nomenclatura e preferência por substratos dos membros da família E-NTPDase em vertebrados. Adaptado a partir de Zimmermann (2001). *Razão de hidrólise NTP:NDP. As NTPDases1 a 3 hidrolisam todos os outros nucleotídeos purícos e pirimidínicos, similarmente ao ATP e ADP.

Por fim, a identificação molecular dos subtipos individuais das NTPDases, com o auxílio da engenharia genética, análises de mutações e a geração de anticorpos específicos não apenas levaram a consideráveis descobertas em relação à estrutura e função dessas enzimas, mas permitiram avanços que têm contribuído para definir funções fisiológicas e patológicas das NTPDases em diversos tecidos.

1.4.3.2 Ecto-5´-nucleotidase/CD73 (Ecto-5´-NT/CD73)

A ecto-5'-NT/CD73 é um homodímero ligado à membrana plasmática através de uma âncora lipídica de glicosilfosfatidilinositol (GPI) e constitui a enzima chave no passo final na degradação de nucleotídeos extracelulares, hidrolisando os nucleosídeos monofosfatados em seus respectivos nucleosídeos e fosfato inorgânico (PI). Dessa forma, é a principal fonte enzimática de adenosina no meio extracelular (Zimmermann, 1992). O controle da sua atividade enzimática é essencial para a manutenção dos níveis extracelulares de adenosina e, consequentemente, dos efeitos mediados via receptores P1 (Figura 5), os quais, no sistema nervoso, resulta principalmente na inibição da liberação de neurotransmissores excitatórios (Brundege & Dunwidie, 1997) enquanto que, no sistema cardiovascular, resulta em vasodilatação e inibição da agregação plaquetária. Tais habilidades tornam a ecto-5'-NT/CD73 um ponto importante de controle o que torna essa enzima um alvo interessante de investigação e de intervenção farmacológica na inflamação.

1.5 Homocisteína

A homocisteína é um aminoácido sulfurado sintetizado a partir da metionina, que é um aminoácido essencial, estando presente abundantemente nas proteínas de origem animal (Selhub, 1999). A homocisteína no plasma sanguíneo está presente em diversas formas: aproximadamente 1% na forma livre (tiol), 80 a 90% ligado à albumina, 10 a 20% combinada com a própria homocisteína ou outros aminoácidos, na forma de dímero (Perla-Kaján et al., 2007). O mau funcionamento de seu metabolismo está associado com muitas desordens, como por exemplo, o aumento de seus níveis no sangue, hiper-homocisteinemia, o qual está associado principalmente a eventos

aterotrombóticos, com uma associação causal, independente de outros fatores de risco para doença arterial (Cattaneo, 1999; Undas *et al.*, 2005; Mohamed *et al.*, 2007). Além disso, nos últimos anos, o aumento dos níveis desse aminoácido no sangue está sendo associado com o aumento de marcadores e processos inflamatórios (Yxfeldt *et al.*, 2003; Schroecksnadel *et al.*, 2003, 2004; Lazzerini *et al.*, 2007).

A homocisteína pode ser formada intracelularmente da desmetilação da metionina proveniente da dieta, no ciclo da metionina (Figura 6). O processo inverso, denominado remetilação, que consiste na formação da metionina a partir da homocisteína requer que esse último aminoácido adquira um grupo metil do *N*-5-metiltetraidrofolato (MTHF), um intermediário no ciclo do folato ou da betaína (presente somente no fígado e rim), através da enzima metionina sintetase (MS), dependente de vitamina B_{12} (cobalamina) (Selhub, 1999; Undas *et al.*, 2005; Jakubowski, 2006).

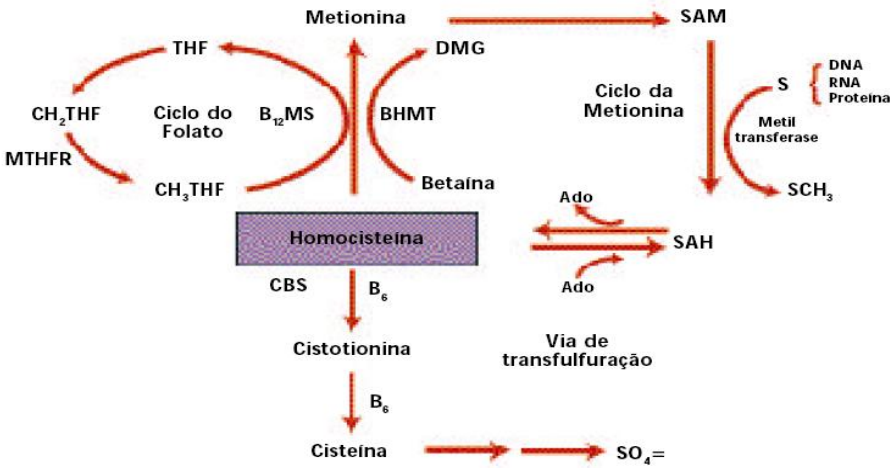

Figura 6. Vias do metabolismo da homocisteína (Adaptado de Jacobsen, 2008).

A metionina é preferencialmente ativada à *S*-adenosilmetionina (SAM), um grupo doador universal de metilas, que transformado em *S*-adenosilhomocisteina (SAH) sofre hidrólise para gerar homocisteína, propagando o ciclo da metionina. A

homocisteina é desviada para o caminho da transulfuração quando a concentração de metionina excede a capacidade do ciclo da metionina, do ciclo remetilação por folato ou quando a síntese de cisteína é requerida. O primeiro passo da reação transulfuração é irreversível e catalisado pela cistationa β sintetase (CBS), a qual é dependente de vitamina B_6 (piridoxina) (Selhub, 1999; Undas *et al.*, 2005; Jakubowski, 2006). Assim, a transulfuração não é importante somente para a síntese de cisteína, mas serve para catabolizar a homocisteína em excesso. A homocisteína é metabolizada intracelularmente e uma proporção muito baixa é liberada na corrente circulatória (Christensen *et al.*, 1999). Portanto, vários são os fatores que podem influenciar o aumento nos níveis plasmáticos de homocisteína e esses podem ser tanto causados por defeito genético (origem primária) (Rozen, 2000) em uma das enzimas, quanto por uma deficiência nutricional de cofatores (vitaminas) da remetilação ou da transulfuração (origem secudária) (Carmel et al., 2002). Em condições normais, o nível plasmático de homocisteína em humanos é baixo (5–15 μM). Quando seus níveis estão acima dos normais, podemos identificar 3 grupos: leve (15-25 μM) e moderado (25-50 μM), que são relacionados principalmente a fatores adquiridos (origem secundária) enquanto, o aumento acima de 100 μM é considerado severo e geralmente está relacionado a defeitos de origem primária, nas enzimas cistationa β sintetase e metileno-tetraidrofolato redutase (Perla-Kaján et al., 2007).

Quando em excesso, a homocisteína rapidamente se auto-oxida no plasma e radicais livres são produzidos (Mohamed *et al.*, 2007; Mohan *et al.*, 2008). Este é o mecanismo comum de patogênese associado à hiper-homocisteinemia. No sistema vascular, os radicais livres acarretam em dano oxidativo ao endotélio (Kanani *et al.*, 1999; Chambers *et al.*, 1999), supressão de óxido nítrico (Durand *et al.*, 2001), proliferação da musculatura vascular lisa (Majors *et al.*, 1997), ativação plaquetária e a

interrupção no balanço normal entre agentes pró-coagulantes e anticoagulantes, favorecendo a formação de trombos (Durand *et al.,* 1996, 1997; Hajjar *et al.,* 1998; Coppola *et al.,* 2000).

Por fim, evidências mostram que em processos inflamatórios como artrite reumatóide e doenças inflamatórias intestinais (Doença de Crohn), a homocisteína pode atuar como uma molécula pró-inflamatória/imuno-estimulatória e cooperar com a injúria e desenvolvimento dessas doenças (Wallberg-Jonsson *et al.,* 2002; Schroecksnadel *et al.,* 2003; Yesilova *et al.,* 2005; Lentz, 2005; Lazzrerini *et al.,* 2007; Chung *et al.,* 2007). A hiper-homocisteinemia costuma estar associada ao aumento da secreção de citocinas pró-inflamatórias que incluem: IL-1β, IL-6, IL-12, IL-18, aumento da expressão de ICAM-1, MMP-9, entre outros (Holven *et al.,* 2003; Mansoor *et al.,* 2004; Su *et al.,* 2005; Gori *et al.,* 2005; Holven *et al.,* 2006; Tso *et al.,* 2006; Cunha *et al.,* 2010). O mecanismo molecular que medeia os efeitos imunes e pró-inflamatórios da homocisteína parecem ser devido a sua capacidade em aumentar a produção de espécies reativas de oxigênio (ROS), presumidamente envolvendo a estimulação de NAP(P)H oxidase e ativação de NF-kappaB (Au-Yeung *et al.,* 2004; Lazzerini *et al.,* 2006).

1.6 Sistema purinérgico e inflamação

A inflamação é desencadeada por 2 tipos de mediadores: células efetoras e fatores solúveis (Barton, 2008; Medzhitov, 2008). A família de células inflamatórias inclui: fibroblasto, células endoteliais, plaquetas, além de macrófagos, neutrófilos, linfócitos e mastócitos. Entretanto, o aumento dessas células em número é irrisório se comparado ao crescimento exponencial da síntese de mediadores inflamatórios solúveis (Barton, 2008; Medzhitov, 2008), dentre esses, os nucleotídeos de adenina e uracila

(ATP e UTP) e nucleosídeos (adenosina) que atuam em receptores P2 e P1 (la Sala, *et al.,* 2003; Khakh *et al.,* 2006; Abbracchio *et al.,* 2007). Além disso, as enzimas que degradam nucleotídeos extracelulares como as NTPDases (CD39) e ecto5´-NT/CD73 também tem uma profunda atividade imunomodulatória (Linden *et al.,* 2001; Dwyer et al., 2007; Yegutkin *et al.,* 2008; Di Virgilio *et al.,* 2009).

A importância dos nucleotídeos extracelulares na "comunicação" (interação) célula-célula tem sido estudada detalhadamente ao longo de anos, principalmente nos sistema nervoso e cardiovascular, enquanto que seu papel no sistema imune é menos conhecido. Porém, a descoberta de ações envolvendo células imunes e o sistema purinérgico despertou um grande interesse na literatura pelo tema.

O ponto central foi a descoberta de uma forte ligação de um dos receptores do tipo P2 ($P2X_7$) na maturação e secreção de uma citocina chave no processo inflamatório: a interleucina-1β (Perregaux *et al.,* 1994; Ferrari *et al.,* 1996; Di Virgilio, 2007). Além disso, foi demonstrado *in vivo* que o ATP está presente em altas concentrações no espaço extracelular durante a inflamação (Idzko *et al.,* 2007; Pellegatti *et al.,* 2008). Após esses acontecimentos, uma completa caracterização foi realizada na expressão de receptores purinérgicos em quase todas as células inflamatórias. Essa varredura não se deteve somente aos receptores ionotrópicos $P2X_{1\text{-}7}$, mas também aos receptores metabotrópicos $P2Y_{1,2,4,6,11,12,13,14}$. O $P2X_7$ tem seu papel nas células efetoras no processo inflamatório mais bem definido, mas outros receptores P2, ($P2Y_2$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ e $P2X_4$), tem revelado uma relação muito próxima com a inflamação. O receptor $P2Y_2$ mostrou recentemente ter um papel crucial na orientação de neutrófilos em uma gradiente quimioatrativo (Chen *et al.,* 2006), enquanto $P2Y_6$ em inflamações intestinais está envolvido na liberação de CXCL8 (também conhecida como IL-8), IL-6 e de MIP2 (Grbic *et al.,* 2008; Bar *et al.,* 2008) Em contribuição a esses achados, uma

descoberta recente mostrou que a CD39 é um marcador imunossupressivo de células Treg Foxp3+ e dessa forma, ressalta as ações do ATP como imunomodulador (Borsellino *et al.*, 2007). Por outro lado, a adenosina induz ações contrárias ao ATP, via receptores P1, e atua mediando angiogênese, regeneração tecidual e a desativação de células imunes (imunossupressão) (Bours *et al.*, 2006).

Portanto, imunomoduladores, como o ATP extracelular e seus metabólitos como a adenosina, atuam como moléculas de sinalização que auxiliam no controle da inflamação e da resposta imune através da regulação na síntese e inibição de citocinas inflamatórias. Dessa forma, o padrão de expressão e as propriedades catalíticas das NTPDases e da Ecto-5´-NT/CD73 influenciam fortemente as ações destas moléculas imunomodulatórias nos seus respectivos receptores purinérgicos e impactando na regulação da síntese de citocinas inflamatórias durante o processo de inflamação.

2. OBJETIVOS

2.1 Objetivo Geral:

Investigar a atividade e expressão das ectonucleotidases em diferentes fenótipos de macrófagos e avaliar os efeitos da homocisteína nessas enzimas em macrófagos e plaquetas.

2.2 Objetivos Específicos:

- ✓ Analisar comparativamente a expressão das ectonucleotidases e dos receptores purinérgicos em macrófagos de camundongos estimulados com LPS (macrófagos classicamente ativados) e IL-4 (macrófagos alternativamente ativados) (item 3.1);

- ✓ Analisar o efeito da homocisteína sobre o fenótipo de macrófagos de camundongos e na expressão das ectonucleotidases e dos receptores purinérgicos (item 3.2);

- ✓ Analisar o efeito da homocisteína *in vitro* e *in vivo* sobre a hidrólise de ATP, ADP e AMP em plaquetas de rato (item 3.3).

3. RESULTADOS

3.1 Artigo 1

DIFFERENTIAL MACROPHAGE ACTIVATION ALTERS THE PROFILE OF E-NTPDase AND ECTO-5'-NUCLEOTIDASE EXPRESSION

Rafael Fernandes Zanin, Elizandra Braganhol, Letícia Scussel Bergamin, Luís Felipe Ingrassia Campesato, Alfeu Zanotto Filho, José Cláudio Fonseca Moreira, Fernanda Bueno Morrone, Maria Rosa Chitolina Schentinger, Angela Terezinha de Souza Wyse and Ana Maria Oliveira Battastini.

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DIFFERENTIAL MACROPHAGE ACTIVATION ALTERS THE PROFILE OF E-NTPDase AND ECTO-5'-NUCLEOTIDASE EXPRESSION

Rafael Fernandes Zanin[1], Elizandra Braganhol[1], Letícia Scussel Bergamin[1], Luís Felipe Ingrassia Campesato[1], Alfeu Zanotto Filho[1], Fernanda Bueno Morrone[2], Maria Rosa Chitolina Schetinger[3], Angela Terezinha de Souza Wyse[1] and Ana Maria Oliveira Battastini[1]*.

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil

[2] Faculdade de Farmácia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brasil

[3]Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, RS, Brasil

***Corresponding author:**

Dra. Ana Maria Oliveira Battastini

Departamento de Bioquímica

Instituto de Ciências Básicas da Saúde

Universidade Federal do Rio Grande do Sul

Rua Ramiro Barcelos, 2600-anexo

Porto Alegre, RS, Brasil - 90035-003

E-mail: abattastini@gmail.com

ABSTRACT

Macrophages are key elements in the inflammatory process, whereas depending on the microenvironmental stimulation they exhibit a pro-inflammatory (classical/M1) or an anti-inflammatory/reparatory (alternative/M2) phenotype. Extracellular ATP can act as a danger signal whereas adenosine, the ATP breakdown product, serves as a negative feedback mechanism to limit inflammation. The local increase in nucleotides communication is controlled by ectonucleotidases, such as ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5′-nucleotidase/CD73 (ecto-5′-NT/CD73). In the present work we evaluated the participation of these enzymes in the resident, LPS (M1 phenotype) and IL-4 stimulated macrophages (M2 phenotype). The results reveal that LPS-stimulated macrophages decreased ATP and AMP hydrolysis followed by a decrease in E-NTPDase1, E-NTPDase3 and ecto-5'-nucleotidase expression compared to IL-4 stimulated and resident cells. In the other hand, stimulation with IL-4 showed a higher ATP hydrolysis and increase of E-NTPDase1 and E-NTPDase3 expression compared to LPS-stimulated and resident macrophages. The results showed also that P1 and P2 purinoreceptors present the same mRNA profile in the studied phenotypes. Therefore, the phenotype differentiation led to a change of nucleotide profile hydrolysis what may drive to a degradation of ATP, a danger signal molecule, to adenosine, a molecule with antiinflammatory and angiogenic properties.

Keywords: Macrophages, phenotype, E-NTPDase, ecto-5'-nucleotidase, ATP, adenosine,

1. INTRODUCTION

Macrophages are key cells involved in the inflammatory process and are characterized by a marked phenotypic heterogeneity depending on their microenvironmental stimulation [1, 2]. These cells exhibit diverse biochemical properties that influence pathobiology with classical/M1 and alternative/M2 polarization representing phenotypic extremes [3]. Classical activation is induced by microbial agents and/or T helper cell type 1 (Th1) cytokines and interferon-γ (IFN- γ), being associated with the production of large amounts of nitric oxide (NO) and proinflammatory cytokines (IL-1β, IL-6, IL-12 and TNF-α), which are involved in cytotoxicity and microbial killing [4, 5]. In contrast, alternative activation is induced by Th-2 cytokines (IL-4 and/or IL-13), and is characterized by antiinflammatory and tissue repair properties [3]. IL-4 stimulates the production of antiinflammatory cytokines such as IL-10 and IL-1R antagonist [6] and inhibits the production of proinflammatory cytokines IL-1β, TNF-α, IL-6 and IL-12 [7, 8], thus reducing inflammation. Moreover, alternative activated macrophages are characterized by an increase in the extracellular matrix remodeling associated with the expression of matrix proteins such as fibronectin, βIGH3, fibrogenesis and a high expression of arginase, which is related to repair properties [9-12].

Macrophages can also respond to endogenous stimuli that are rapidly generated following injury [1]. Nucleotides and nucleosides are currently considered as true inflammatory mediators [13-16]. The concentration of nucleotides/nucleosides in the extracellular space is maintained at low levels but accumulation of these molecules may

occur in some situations such as mechanical stress, cell injury and inflammation. Extracellular ATP can act as a danger signal molecule initiating an innate immune response. For example, this nucleotide induces the macrophages to release a repertory of pro-inflammatory cytokines such as IL-1β and IL-6 and superoxide, which are essential for damage agent dissolution [17-21]. In contrast, the ATP breakdown product adenosine serves as a negative feedback mechanism, limiting inflammation by suppress the actions of virtually all immune cells[21, 22].

Biological effects of extracellular nucleotides/nucleosides are evoked by activating transmembrane receptors of the P2 and P1 family [23], which are expressed by monocytes/macrophages. Primary monocytes express at mRNA level the ion-channel $P2X_{1,4,5,7}$ and the G-protein-coupled $P2Y_{1,2,4,6,11–13}$ receptors, while macrophages express the same receptor subtypes except $P2Y_{13}$ [24, 25]. $P2Y_1$, $P2Y_2$, $P2X_4$ and $P2X_7$ receptors have been detected in macrophages at protein level [24-26]. All four (A_1, A_{2A}, A_{2B} and A_3) adenosine receptors are expressed by both monocytes and macrophages [21, 22].

ATP and UTP mediated cell communication is controlled by ectonucleotidases, such as ectonucleoside-triphosphate diphosphohydrolase (E-NTPDase) and ecto-5′-nucleotidase/CD73 (ecto-5′-NT/CD73) [27, 28]. The E-NTPDases efficiently hydrolyze nucleoside 5’-triphosphates and diphosphates (physiological ATP, UTP, ADP and UDP) to its respective nucleoside-5’-monophosphate with considerable difference in their preference for individual type of nucleotide. In mammals, eight related enzymes named NTDPase1–8 have been cloned and characterized. Four of the NTPDases are typical cell surface-located enzymes with an extracellularly facing catalytic site (NTPDase1, 2, 3, 8). E-NTPDase1/CD39 hydrolyzes ATP and ADP with comparable rates producing almost

directly AMP; E-NTPDase2 exhibits high preference for ATP and therefore, could favor extracellular ADP accumulation,E-NTPDase3 and 8 reveal an intermediary rate preference for ATP. AMP produced by E-NTPDase activity is further hydrolyzed to adenosine by ecto-5'-nucleotidase/CD73 (ecto-5'-NT/CD73) [27-29]. E-NTPDase1/CD39 and ecto-5'-NT/CD73 are distributed on the membrane of different blood cells, such as leukocytes [30, 31], endothelial cells [31] and platelets [32] being involved in the inhibition of platelet recruitment and thrombus formation [33] leukocyte migration [34] and immunessupressive functions [35].

Hence, considering the importance of the macrophage spectrum activation and the purinergic signaling in the innate immune responses, we hypothesize that the ectonucleotidases, by modulating the P2/P1R activation, may participate in the macrophage phenotypic changes. In agreement, in the present work we show that NTPDases and ecto-5'-NT/CD73 are diffentially expressed in resident, classical and alternative activated macrophages. The participation of the ectonucleotidase pathway along the differential macrophage activation process is further discussed and may give an insight about the role of purinergic signaling in the phenotypic modulation of immune cells.

2. MATERIALS AND METHODS

2.1 Animals and Reagents

Swiss male mice, 6-8 weeks-old, were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room temperature (22 ± 2°C). The

mice had free access standard laboratory mice chow and water. The animal handling and experiments were performed in accordance with the international guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Macrophages activation

Peritoneal macrophages were collected by lavage of the peritoneal cavity with 5 mL of sterile RPMI-1640 medium without fetal bovine serum (FBS). The cells were washed twice with sterile Phosphate Buffered Saline (PBS) and suspended in RPMI without FBS. The cells obtained were transferred to 6, 24 or 48 multiwell plates and allowed to attach for 30 min. Unattached cells were washed out with RPMI without FBS. The attached cells, mainly peritoneal macrophages, were used for the experiments thereafter. Macrophage were evaluated by microscopic examination of the culture wells after May-Grrunwald and Giemsa stains, indicating macrophage purity higher than 80%, which were confirmed with CD11b Ab.

The obtained macrophages were stimulated for 24 h in complete medium (RPMI plus 10% FBS) with LPS (100 ng/mL) or IL-4 (10 ng/mL) (Sigma) for the generation of classically or alternative macrophage activation, respectively. Resident macrophages were maintained in RPMI/10% FBS.

2.3 Arginase and Nitrite Assays

Arginase activity in cell lysates was measured based on the conversion of L-arginine to L-ornithine and urea according to the technique described by Corraliza and collaborators [36] with minor modifications. Briefly, cells were lysed for 30 min with 40

µL of 0,1% Triton-X-100. Thirty microliters of 25 mM Tris-HCl, pH 7.4 and 10 µL of 10 mM $MnCl_2$ were added and the enzyme was heat-activated for 10 min at 56^{o}C. Similar amounts of sample (40 µL) and 0.5 M L-arginine (pH 9.7) were mixed and incubated for 1 h at 37^{o}C. The reaction was stopped by adding 400 µL of H_2SO_4 (96%), H_3PO_4 (85%), H_2O (1/3/7, v/v/v). The urea concentration was measured at 540 nm after the addition of 8 µL of α-isonitropropiophenone 6%, followed by heating at 95^{o}C for 30 min. Values were compared with a standard curve of urea concentration.

Nitrite concentrations were measured using the Greiss reaction [37]. In brief, 200 µL of the tested cell medium were incubated with 100 µL of 1% sulfanilamide and 100 µL of 0.3% N-1-naphthylethylenediamine dihydrochloride at room temperature for 5 min. Nitrite was quantified by spectrophotometry at 540 nm using sodium nitrite as standard.

2.4 Determination of cytokine release

Cell medium was collected and the tumor necrosis factor (TNF-α) and interleukin-10 (IL-10) levels (; were determined by enzyme-linked immunoabsorbent assay (ELISA), according to the manufacturer's instructions (R&D Systems).

2.5 Ectonucleotidase assay

To determine the ATPase, ADPase and AMPase activities, 48 multiwell plates containing macrophages cells were washed three times with incubation medium in absence of nucleotides. The enzymatic reaction was started by the addition of 200 µL of incubation medium containing 2 mM $CaCl_2$ (2mM of $MgCl_2$ to AMPase assay), 120 mM

NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4 and 2 mM of ATP, ADP or AMP as substrates, at 37^{o}C. After 10 min of incubation, the reaction was stopped by collecting an aliquot of the incubation medium and transferring it to a pre-chilled tube containing trichloroacetic acid (final concentration 5% w/v). The release of inorganic phosphate (Pi) was measured by the malachite green method [38], using KH_2PO_4 as a Pi standard. Controls to determine nonenzymatic

Pi release were performed by incubating the cells in the absence of the substrate or the substrate in the absence of the cells. All samples were run in triplicate. The protein concentration was measured by the Coomassie Blue method using bovine serum albumin as standard [39]. Specific activity was expressed as nmol Pi released/min/mg of protein.

2.6 Analysis of extracellular ATP and AMP metabolism by HPLC

The cells were incubated as described above, except that ATP or AMP concentrations were 100 µM. To stop the reaction, an aliquot of the incubation medium was transferred to an pre-chilled tubeand centrifuged at 4°C for 30 min at 16,000 *g*. Aliquots of 40 µL were applied to a reverse phase HPLC system using a C18 Shimadzu column (Shimadzu, Japan) with absorbance measured at 260 nm. The mobile phase was 60 mM KH_2PO_4, 5 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol as described [40]. Retention times were assessed using standard samples of ATP and its metabolites. The non-enzymatic hydrolysis of the ATP and AMP were consistently less than 5% of degradation. Cells incubated in incubation nucleotide free medium did not present any detectable peak.

2.7 RT-PCR and Real Time PCR

The RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, California, USA). One μg of total RNA were added to each cDNA synthesis reaction using the SuperScript-III RT pre-amplification system (Invitrogen, cidade, estado). The PCR reactions were performed in 25 μL of the reaction mixture containing 1 μL cDNA, 10 pmol of the primer in the supplied PCR mix buffer (Platinum PCR Supermix, Invitrogen). After initial denaturation for 5 min at 95°C, the amplifications were carried out for 35 cycles of denaturation at 94°C for 45 seg, annealing at primer specific temperature for 45 seg and extension at 72°C for 45 seg. The PCR was ended by 7 min incubation at 72°C. The same program was used for the amplification of the reference gene β-actin. Sequences of primers are listed in Table 1. The PCR products were separated by 1.5 % agarose gel electrophoresis and visualized with SYBRGolg (Molecular Probes).

Real-time PCR was carried out in the Applied-Biosystem Step One Plus cycler using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, xxxx) following the manufacturer's instructions and performed in triplicate. Total RNA and cDNA were generated as described above. The selected primers used for real time PCR were the same used in RT-PCR analysis. All results were analyzed by the $2^{-\Delta/\Delta CT}$ method [41]. The β-actin was used as the internal control gene for all relative expression calculations.

2.8 Flow Cytometry

The expression of selected surface proteins by peritoneal macrophages was evaluated by cytometry using the following antibodies (Ab): guinea pig polyclonal anti-mouse NTPDase1/CD39 (mN1-1_c (I_4, I_5)), rabbit polyclonal anti-mouse NTPDase2

(mN2-36_L), guinea pig anti-mouse NTPDase3 (mN3-$1_c I_4$) and rabbit anti-rat ecto-5`-nucleotidase/CD73 (rNu 9_L, $I_{4,}5$). Briefly, the cells were incubated for 30 min with the above primary Ab diluted in PBS, 1% FBS, 0.1% sodium azide (PFA), and, when necessary, with secondary FITC-conjugated goat anti-rabbit IgG Ab (Invitrogen, xxx) or Alexa 488-conjugated goat anti-guinea pig IgG Ab (Invitrogen, xxx) for 30 min, with a minimum of two washes with PFA after each incubation. Cell surface fluorescence was measured with FACSCalibur Flow Cytometer (BD Biosciences).

2.9 Statistical analysis

Data were expressed as mean ± S.D. and were subjected to one-way analysis of variance (ANOVA) followed by Tukey post-hoc test (for multiple comparisons). Differences between mean values were considered significant when $P<0.05$.

3. RESULTS

3.1 Characterization of activated macrophages

In order to define the phenotypic extremes of macrophage activation primary macrophages were stimulated with LPS or IL-4 and the arginase/iNOS activities, Ym1 and FIZZ1 mRNA expression and cytokine production were evaluated.

iNOS is up-regulated in response to inflammatory stimuli as macrophages shift towards the classical/M1 phenotype and become involved in the initiation of the immune response, while the expression of arginase is induced by Th2-type cytokines characterizing the alternative/M2 phenotype [2, 3]. Figure 1A shows that, as expected, cells stimulated in culture with LPS exhibited an increase in the iNOS activity when

compared to resident and IL-4 treated macrophages. In contrast, IL-4 treatment induced an increase in arginase activity (Fig. 1B).

To understand the spectrum of macrophage polarization, authors have studied genes that are expressed by different macrophage phenotypes. It has been shown that the expression of Ym1 and FIZZ1 is strongly induced in alternative/M2 activated macrophages *in vivo* and *in vitro* in murine experimental models [3]. Accordingly, macrophages stimulated with IL-4 exhibited a strong induction of FIZZ1 and Ym1 expression when compared to resident and LPS-induced macrophages (Figure 1B). Finally, the macrophage activation was characterized by the production of inflammatory cytokines. The results showed that LPS-stimulation induced a expressive increase in TNF-α release when compared to resident and IL-4 treated macrophages.In contrast, macrophages IL-4 stimulatedproduced higher levels of IL-10 in relation to resident and LPS treated macrophages (Figure 1C). Taken together, these results confirm that the macrophages used in our experiments are differenced into two phenotypes: macrophages classically activated by LPS (M1) and alternatively activated by IL-4 (M2), and this protocol were applied to further assays described below.

3.2 *Characterization of P1, P2 receptors and ectonucleotidases expression in classical/M1 and alternative/M2 activated macrophages*

Since extracellular nucleotides are associated to immune/inflammatory responses through purinergic receptor activation, the P2XR and P2YR mRNA expression by the macrophages after phenotypic differentiation was analyzed by RT-PCR. As shown in Table 2, no difference was observed on purinergic receptor expression in classical/M1

and alternative/M2 activated-macrophages. Considering that the availability of extracellular adenine nucleotides is controlled by the conjugated action of E-NTPDases and ecto-5´-NT/CD73, the mRNA expression of these ectonucleotidases in macrophages differentially activated was also evaluated by RT-PCR.. The analysis of resident, LPS or IL-4 stimulated macrophages showed the presence of E-NTPDase1, E-NTPDase3 and ecto-5'-NT/CD73 mRNA transcripts, while E-NTPDase2 and 8 were absent (Table 2).

3.3 Differential ectonucleotidase expression in classical/M1 and alternative/M2 activated macrophages

The differential capacity of resident, LPS or IL-4 stimulated macrophages to hydrolyze extracellular ATP, ADP and AMP was investigated. LPS-stimulated macrophages exhibited a decrease in the ATPase (20 %) and AMPase (54 %) activities when compared to resident macrophages, whereas the ADP hydrolysis was not altered (Figure 2). . In contrast, IL-4-stimulated macrophagesshowed a significant increase in ATP (~20 %) and ADP (~25 %) hydrolysis, while it was not observe any alteration in AMP hydrolysis in comparison to resident macrophages. It is important to note that the ectonucleotidase activities were higher in IL-4-stimulated macrophages when compared to LPS-treated cells, suggesting that in alternative/M2 macrophages the nucleotide pathway is directed trough the generation of anti-inflammatory adenosine.

The extracellular nucleotide metabolism analysis by HPLC confirmed the enzyme activity pattern obtained with malachite green method (Figure 3A, B and C). In the LPS-stimulated macrophages, ATP was slowly metabolized along the 120 min of analysis, being converted to ADP and AMP (Fig. 3B). The evaluation of AMP metabolism

indicated a low AMPase activity in these cells and reinforces the idea that extracellular adenosine generation is decreased in LPS exposed macrophages (Fig. 4B).

Confirming the higher ATPase activity exhibited by IL-4 stimulated macrophages, at 20 min incubation ATP is almost completely converted to AMP with a transient ADP accumulation (Fig. 3C). The analysis of AMP metabolism also showed a sustained level of adenosine in the extracellular medium of IL-4 activated macrophages from 40 min to 120 min, although the inosine level were maintained along of the incubation (Fig. 4C). These results, reinforce the evidence that adenosine may be determinant on the macrophages switch phenotype.

Considering the differential nucleotide metabolism pattern presented by resident, classical/M1 and alternative/M2 macrophage activation, the expression of E-NTPase1, E-NTPDase3 and ecto-5'-NT/CD73 at mRNA and protein levels were evaluated. In agreement with the decreased ATP metabolism observed in classical/M1 activated macrophages, the E-NTPDase1, E-NTPDase3, just at protein level, and ecto-5'-NT/CD73 expression was decreased in these cells when compared to resident macrophages (Fig. 5; Fig. 6A and B). The IL-4 stimulation of macrophages promoted an increase in E-NTPDase1, E-NTPDase3 and ecto-5'-NT/CD73 at protein and mRNA level, respectively (Fig. 5; Fig. 6A and B), which is consistent with the ATP extracellular metabolism exhibited this alternative/M2 phenotype. Thus, the three macrophages populations express distinct levels of these molecules, which may be important to modulate the purinergic receptor activation during the macrophage switch in ongoing inflammatory process.

4. DISCUSSION

Macrophages play key roles in the regulation of inflammation and wound healing and are exquisitely sensitive to their microenvironment. Depending on particular exogenous or endogenous stimuli, they can modulate expression of an array of cytokines and induce to differentiate into cells that either exacerbate or inhibit inflammation. Among these stimulus, extracellular nucleotides (ATP and UTP) and nucleosides (adenosine) acting at P2 or P1 purinergic receptors [16] and thereby enzymes that degrade extracellular nucleotides, such as E-NTPDases and ecto-5`-nucleotidase, have also a crucial immunomodulatory activity [42].

In the present study we differentiated *in vitro* peritoneal macrophages into two phenotypes: a pro-inflammatory phenotype named classical/M1stimulated by LPS and anti-inflammatory phenotype named alternative/M2 stimulated by IL-4 in order to characterize the participation of the ectonulceotidases in these biological processes. First we characterized these distinct subpopulation by evaluating biochemical parameters through arginine metabolism, mRNA expression profiles and cytokine production. Classically/M1 activated macrophages exhibited increased iNOS and reduced arginase activities. By contrast, alternatively/M2 activated macrophages showed decreased iNOS activity and increased arginase activity, while in resident macrophages both arginase and iNOS activity were low. Data from the literature demonstrate that high expression of genes FIZZ1 and Ym1 constitute useful markers for the identification of alternatively activated macrophages both *in vitro* and *in vitro* [43, 44]. Our data showed a high expression of these markers only to alternatively/M2 activated macrophages, confirming the alternative phenotype of M2 cells. At last, the results showed different cytokine

production in the classical/M1 and alternative/M2 phenotype. As expected, the classical/M1 phenotype produced higher pro-inflammatory cytokine TNF-α than alternative/M2 phenotype whereas the anti-inflamatory cytokine IL-10 was higher in M2 than M1 phenotype. Taking together the results presented in the Figure 1 ensure that the macrophages were differentiated into two extreme phenotypes M1 and M2.

In a previous study Levésque and collaborators [45] have shown the enzymatic presence of E-NTPDase1 in thioglycollate-elicited macrophages. In accordance with this result we demonstrated that the E-NTPDase1 was present in resident, LPS and IL-4 stimulated macrophages with different pattern of distribution. In addition, we also reveal the presence of E-NTPDase3 and ecto-5'-nucleotidase in mRNA and protein level.

The LPS-activated macrophages presented ATPase reduction of ~20% in relation to resident and 40% of reduction when compared to macrophages stimulated with IL-4. Surprisingly, there was no alteration in ADP hydrolysis in LPS-stimulated macrophages in relation to resident cells. These data were confirmed by results of HPLC, which demonstrated an increased ATP breakdown in resident and IL-4 stimulated macrophages when compared to LPS stimulated cells. It was also verified a reduction in mRNA level (Figure 5) accompanied of a diminishing in the protein expression of E-NTPDase1 and E-NTPDase3 in LPS- treated cells (Figure 6). Previous studies demonstrated loss of NTPDase1 activity in endothelium cells after exposure to LPS [46]. The reduction in NTPDase1 activity can be explained by alterations in the membrane structure due to the LPS-evoked inflammation, since NTPDase1 activity is sensitive to changes in its transmembrane domains and to changes in the properties of the membranes in which they are embedded [47, 48] and LPS induces inflammation which leads to alterations of

membrane structure [49, 50]. This explanation can be expanded to other E-NTPDases, since these enzymes are also sensitive to transmembrane changes. Therefore, our result can be, in part, justified by LPS membrane perturbation but translational and/or posttranslational modifications must be involved in the LPS activation. Moreover, the transient reduction of ATP hydrolysis in the pro-inflammatory phenotype macrophages (M1) should be beneficial to stimulate ATP mediated pathogen clearance. In addition, pro-inflammatory P2X7 receptors appear to be up-regulated in macrophages following classical activation by IFNγ, IL-1, TNFα and LPS [51-53], probably sensitizing macrophages to pro-inflammatory effects of ATP at high levels. Eventually, in macrophages stimulated with IL-4 the outcome showed that ATPase and ADPase activities increased in relation to resident and macrophages stimulated with LPS. Additionally, mRNA and protein expression of E-NTPDase1 and NTPDase3 were increased in this phenotype. In summary higher ATP/ADPase activity in IL-4 stimulated macrophages drive the ATP rapidly to generation of adenosine in this phenotype.

The extracellular adenosine is a metabolite produced, mainly by breakdown of ATP, which is elevated during the inflammation process and is associated to anti-inflammatory and regeneration actions in immune cells [22]. The present results demonstrated that the LPS-induced a decrease in the ecto-5'-NT/CD73 activity in relation to resident and IL-4 stimulated macrophages accompanied by a reduction in the expression of mRNA (Figure 5) and protein level (Figure 6) on the surface of the cells. Experiments have demonstrated that blockade of NF-κB increased the basal activity of ecto-5'-NT/CD73 in endothelial cells (HUVEC) [54]. The stimulation of macrophages by LPS results in the activation of several signaling pathways such as NF-κB, which may

suppress the transcription of the gene encoding ecto-5'-NT/CD73. This notion is supported by studies that have demonstrated that anti-inflammatory effects of methotrexate results from the activation of ecto-5'-NT/CD73 through suppression of NF-κB [55]. Besides this, the reduction in AMP hydrolysis was accompanied by the loss of ecto-5'-nucleotidase on cell surface, suggesting that enzymatic activity is proportional to the abundance of ecto-5'-NT/CD73 protein on cell surface. In fact, our results demonstrated that the loss in the ecto-5'-nucleotidase activity occurred in molecular and protein level. Another interesting result observed on macrophages stimulated with IL-4 was the sustained levels of adenosine produce after AMP hydrolysis. This result must be due to two main factors: i) a higher expression and activity of ecto-5'-NT/CD73 (AMP → ADO) in relation to LPS-induced cells and ii) it should be the result of the decrease in the adenosine degradation to inosine. Thesecond hypothesis is not probable in our conditions because the inosine levels were maintained constant in three different populations of macrophages. Although, inosine has been suggested to participate in inflammatory process, there is a lack of evidence about it [56, 57]. In contrast the role of adenosine and its receptors in the control of immune response, including macrophage activation, it is well established [21, 22]. In this scenario, we can suggest that the sustained levels of adenosine in alternatively/M2 macrophages would be more relevant to the anti-inflammatory and regeneration actions of M2 phenotype macrophages.

Finally, we showed here that the P1 and P2 purinoreceptors revealed the same mRNA profile in the three studied phenotypes. Then, these evidence lead to deduce that the expression change profile of E-NTPDase1, E-NTPDase3 and ecto-5'-nucleotidase enzymes in macrophages during phenotypic differentiation must extent purinergic

signaling to a purinergic cascade that leads to a progressive decrease in nucleotide concentrations and an increase in nucleoside (adenosine) availability. Therefore, such changes in ectoenzyme functionality might allow macrophages to adjust the outcome of the purinergic cascade on their cell surface in order to fine-tune their effectors functions during the inflammatory event.

5. ACKNOWLEGEMENTS

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7. LEGENDS

Figure 1. Phenotype characterization of resident, LPS or IL-4- stimulated macrophages. **(A)** Arginase and iNOS activities: Arginase activity was evaluated by measuring the formation of urea from arginine in activated macrophages. iNOS activity was estimated by the NO^{2-} (nitrite) accumulation in the supernatant of cultured cells. *Significantly different from the two other groups ($p<0.001$). **(B)** FIZZ1 and Ym1 expression in stimulated macrophages was evaluated by qPCR. Expression was normalized to β-actin signals as described in Material and Methods. *Significantly different from resident and LPS-stimulated macrophages ($p<0.001$). **(C)** TNF-α and IL-10 cytokines were measured from supernatants of macrophage cultures. *Significantly different from stimulated and resident macrophages ($p<0.001$). Data show mean ± SD of at least three independent experiments. Significant difference between groups was determined by ANOVA, followed by Tukey's test.

Figure 2. ATP, ADP and AMP hydrolysis in macrophages after phenotype differentiation. The data represent mean ± S.D. (n=5) with pooled macrophages from 6 to 8 mice *per* experiment carried out separately. Data were analyzed by ANOVA, followed by Tukey's test. *Significantly different from resident macrophages; #Significantly different from LPS-stimulated macrophages ($p<0.05$). represent significant statistical between IL-4 and LPS, considering $P<0.05$ as significant.

Figure 3. Metabolism of extracellular ATP by macrophages after phenotype differentiation. Resident macrophages (A), LPS-induced macrophages (B) and IL-4 (C) were incubated in 48 well plates 100 μM of ATP in 200 ml of incubation medium as described in material and methods. An aliquot of the supernatant was withdrawn at 0, 20, 40, 60 and 120 min and the presence of ATP, ADP, AMP and AMP breakdown were determined after separation by HPLC. Data are mean±SD values from three experiments in triplicates.

Figure 4. Metabolism of extracellular AMP by macrophages after phenotype differentiation. Resident macrophages (A), LPS-induced macrophages (B) and alternatively activated macrophages (C) were incubated in 48 well plates 100 μM of AMP in 200 μl of incubation medium as described in material and methods. An aliquot of the supernatant was withdrawn at 0, 20, 40, 60 and 120 min and the presence of AMP, adenosine (ADO) and inosine (INO) were determined after separation by HPLC. Data are mean±SD values from three experiments in triplicates.

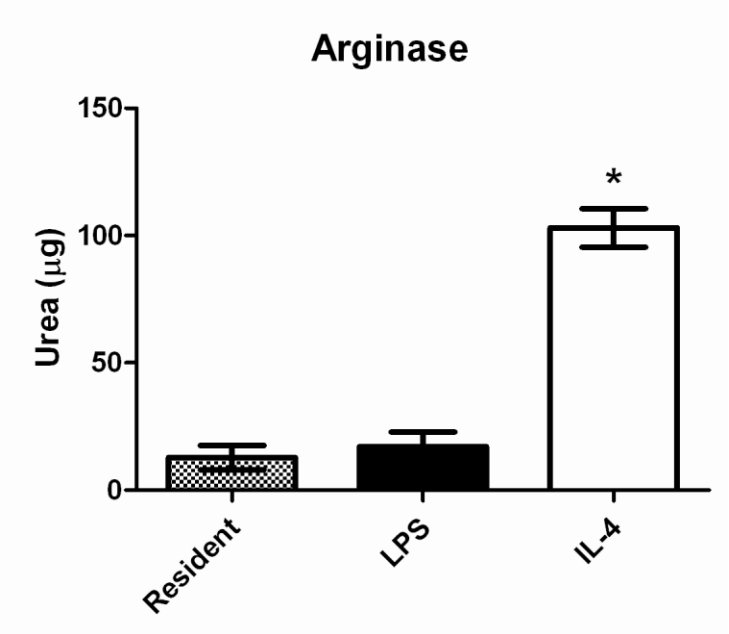
Figure 5. E-NTPDase1, E-NTPDase3 and ecto-5'-nucleotidase expression was quantified by qPCR. The total the amount mRNA were normalized to β-actin signals and expressed as $2^{-\Delta/\Delta CT}$. Data show mean±SD for real time PCR experiments performed in triplicate with RNA purified from three independent experiments with pooled macrophages from 8 to 10 mice per experiment. Macrophages stimulated with LPS and IL-4 were compared to resident macrophages (*) $p<0.001$, and (#) $p< 0.01$ macrophages stimulated IL-4 compared to LPS, two-way ANOVA with Tukey's post-hoc test.

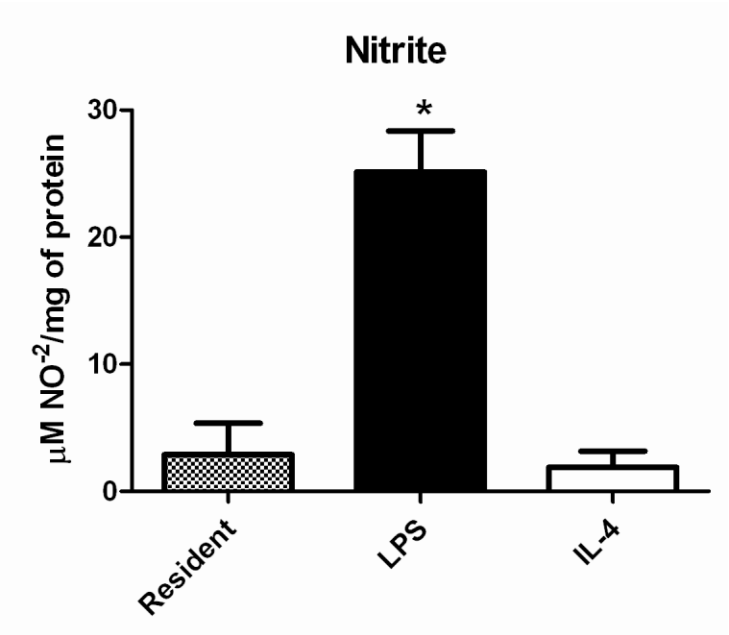
Figure 6. Activated macrophages express different protein levels of E-NTPDases and ecto-5`-nucleotidase. Flow cytometry profiles for (A) E-NTPDase1 E-NTPDase3 (B) ecto-5`-nucleotidase on macrophages 24 h after stimulation. Macrophages were primed with LPS or IL-4 or left unstimulated (resident). Figures are representative of at least three independent experiments with pooled macrophages from 4 to 6 mice per experiment. (*) p< 0,05 Indicated changes in expression when compared against resident and (#) p< 0.05 indicated significance difference of macrophages stimulated IL-4 compared to LPS, two-way ANOVA with Tukey's post-hoc test. MFI – Mean Fluorescence Intensity

8. FIGURES

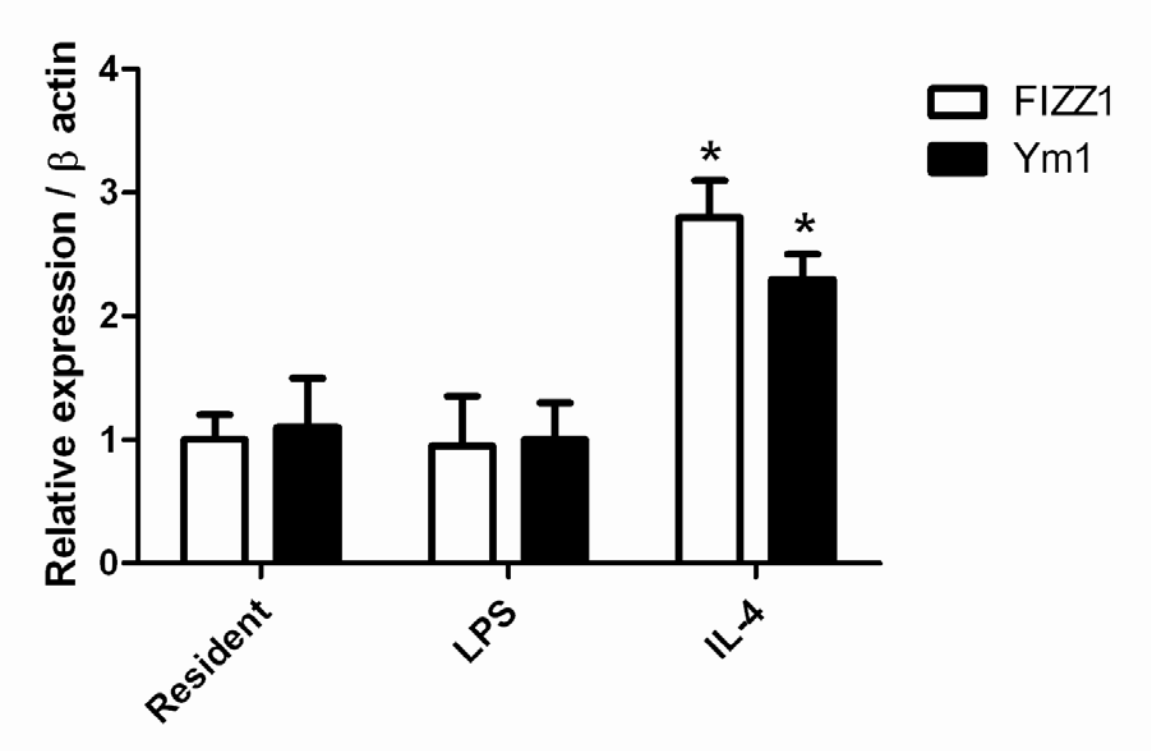
FIGURE 1

(A)

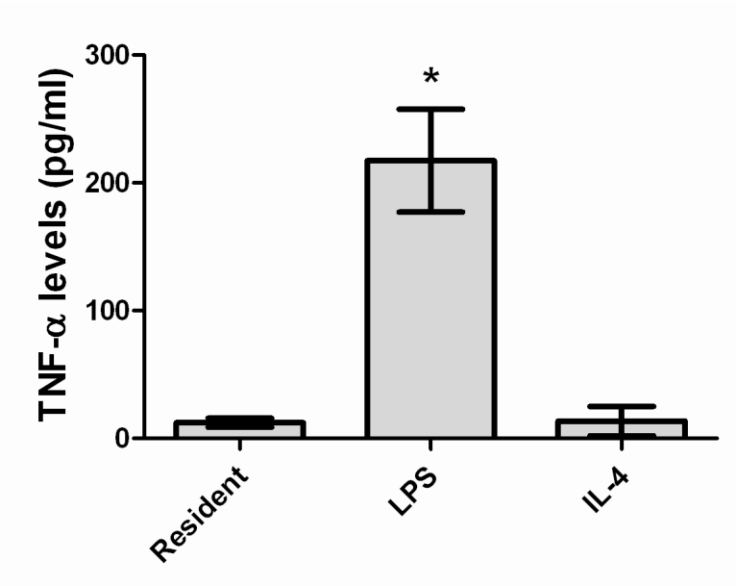




(B)


Relative expression / β actin
4
3
2
1
0
*
*
FIZZ1
Ym1
Resident
LPS
IL-4

(C)


TNF-α levels (pg/ml)
300
200
100
0
*
Resident
LPS
IL-4

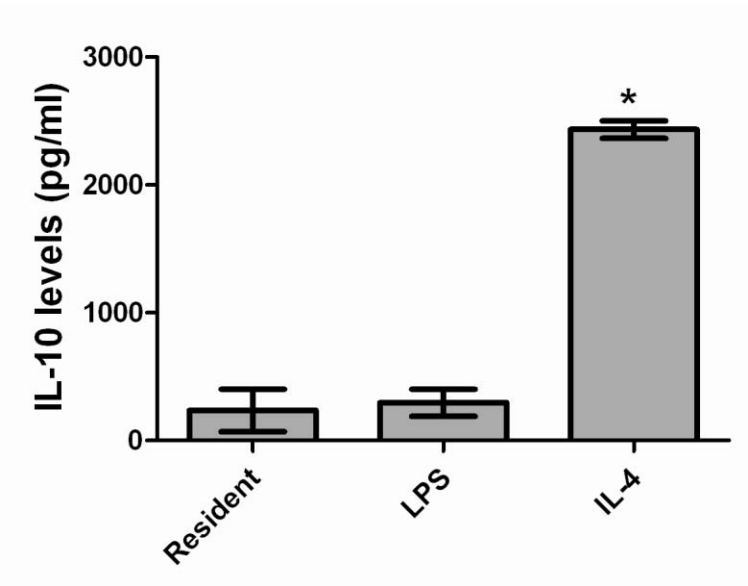

IL-10 levels (pg/ml)
3000
2000
1000
0
*
Resident
LPS
IL-4

FIGURE 2

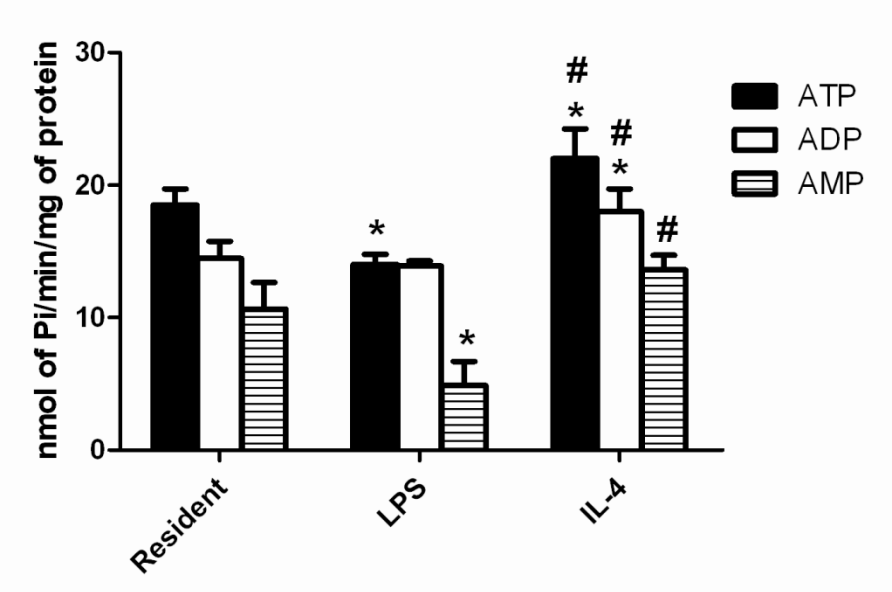
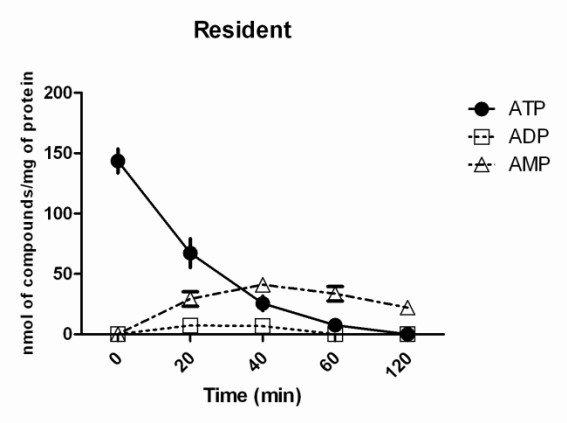
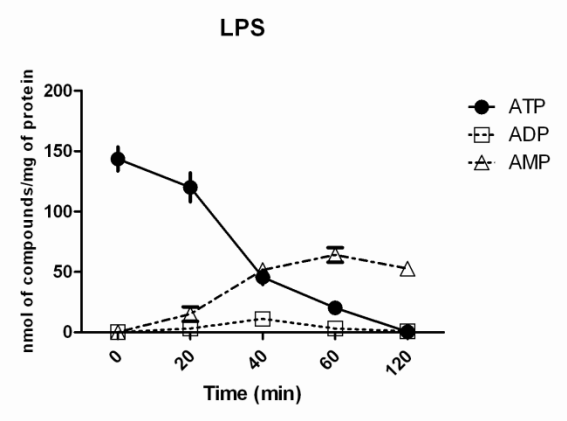

nmol of Pi/min/mg of protein
30
20
10
0
ATP
ADP
AMP
Resident
LPS
IL-4
*
#

FIGURE 3

(A)


Resident
nmol of compounds/mg of protein
200
150
100
50
0
0
20
40
60
120
Time (min)
ATP
ADP
AMP

(B)


LPS
nmol of compounds/mg of protein
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150
100
50
0
0
20
40
60
120
Time (min)
ATP
ADP
AMP

(C)

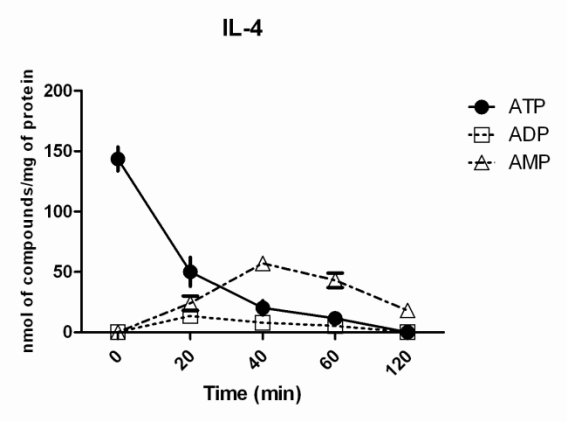
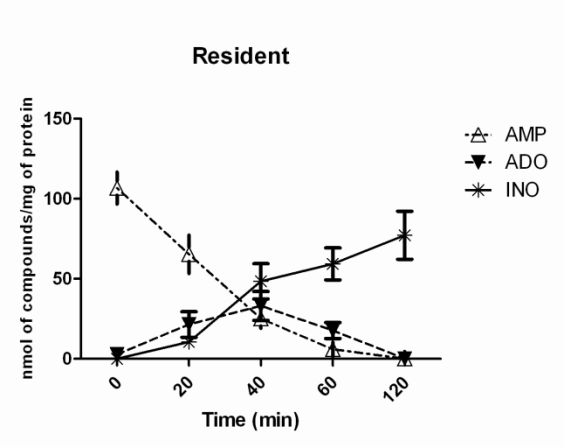
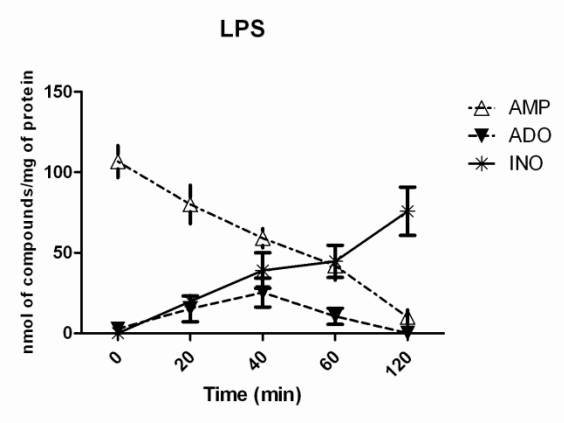

IL-4
nmol of compounds/mg of protein
200
150
100
50
0
0
20
40
60
120
Time (min)
ATP
ADP
AMP

FIGURE 4

(A)


Resident
nmol of compounds/mg of protein
150
100
50
0
0
20
40
60
120
Time (min)
AMP
ADO
INO

(B)


LPS
nmol of compounds/mg of protein
150
100
50
0
0
20
40
60
120
Time (min)
AMP
ADO
INO

(C)

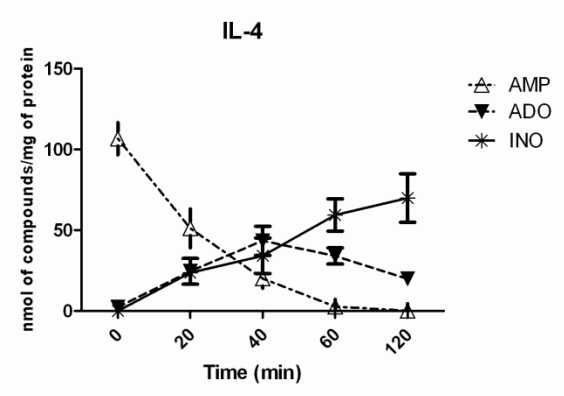

IL-4
nmol of compounds/mg of protein
150
100
50
0
0
20
40
60
120
Time (min)
AMP
ADO
INO

FIGURE 5

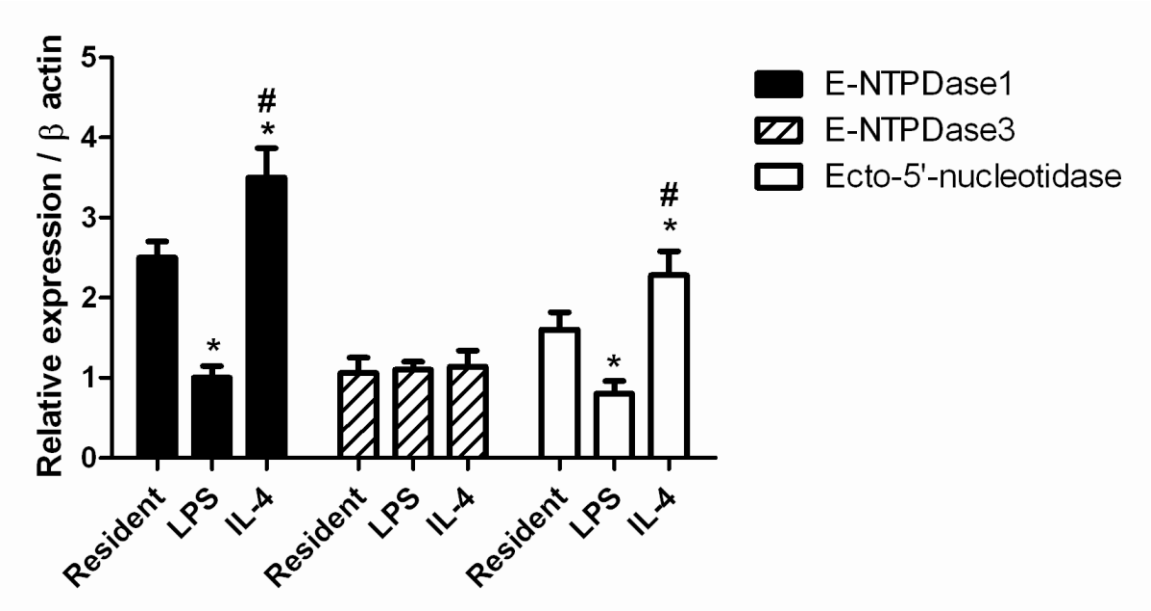
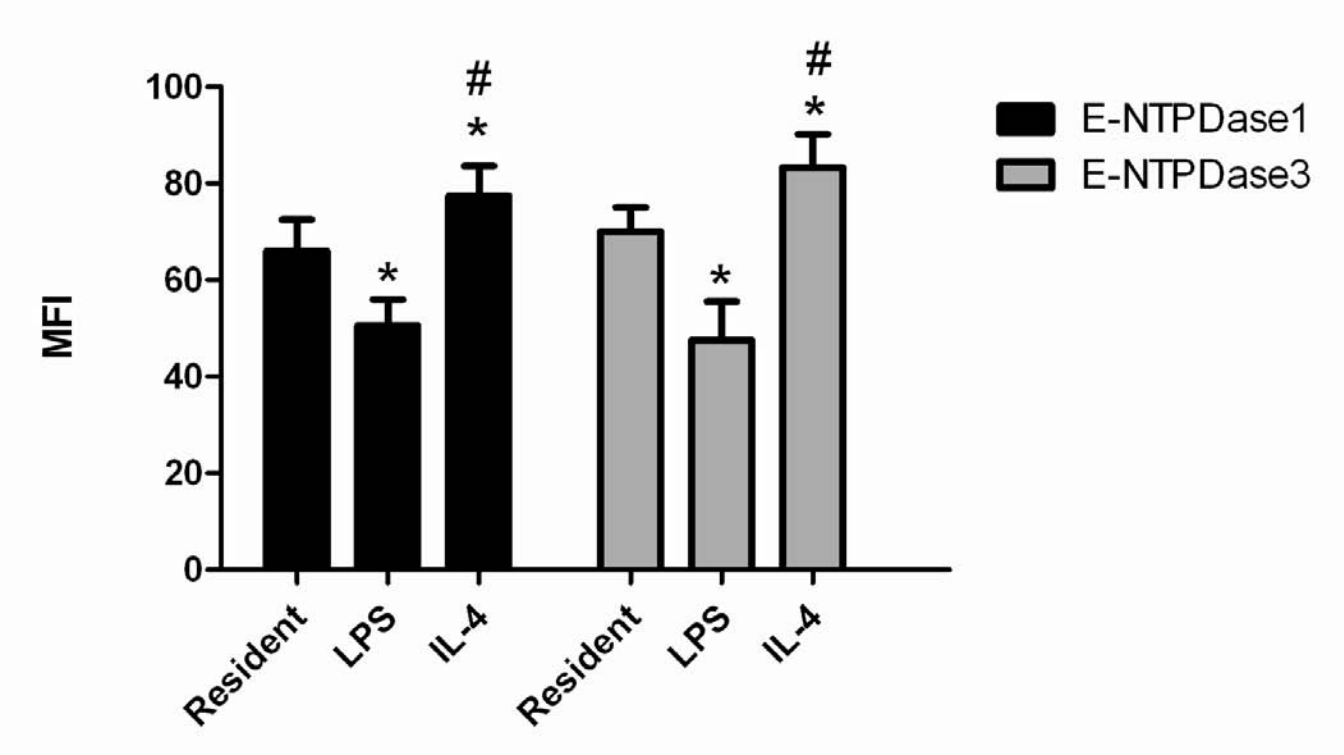

Relative expression / β actin
5
4
3
2
1
0
#
*
*
#
*
*
Resident
LPS
IL-4
Resident
LPS
IL-4
Resident
LPS
IL-4
E-NTPDase1
E-NTPDase3
Ecto-5'-nucleotidase

FIGURE 6

(A)


MFI
100
80
60
40
20
0
#
*
*
*
#
*
Resident
LPS
IL-4
Resident
LPS
IL-4
E-NTPDase1
E-NTPDase3

(B)

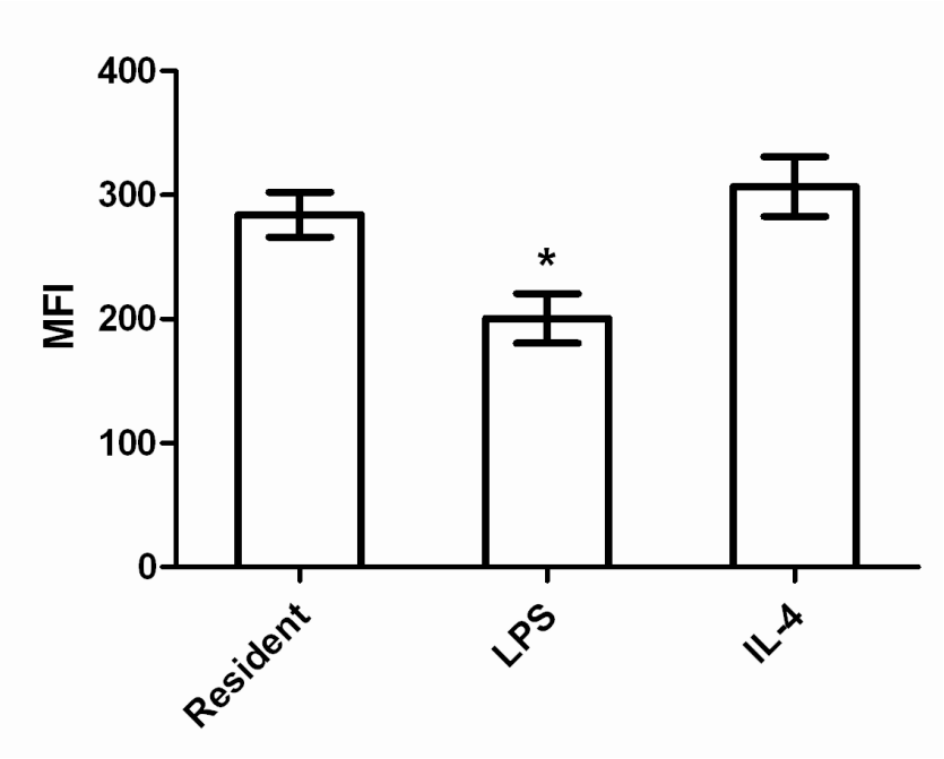


TABLE 1. Primers for purinergic receptors and ectonucleotidases enzymes used at RT-PCR and real time PCR experiments.

Primer	Sense	Antisense
P2X1	5`-AAGGTCAACAGGCGCAACC-3`	5`-AACACCTTGAAGAGGTGACG-3`
P2X2	5`-GTGCAGAAAAGCTACCAGG-3`	5`-GGATGGTGAAATTTGGGGC-3`
P2X3	5`-GCTGCGTGAACTACAGCTC-3`	5`-ACTGGTCCCAGGCCTTGTC-3`
P2X4	5`-CTTGGATTCCGGATCTGGG-3`	5`-GGAATATGGGGCAGAAGGG-3`
P2X5	5`-GCACCTGTGAGATCTTTGC-3`	5`-TCGGAAGATGGGGCAGTAG-3`
P2X6	5`-CAGGACCTGTGAGATCTGG-3`	5`-TCCTGCAGCTGGAAGGAGT-3`
P2X7	5`-TCCCTTTGCAGGGGAACTC-3`	5`-GTACGGTGAAGTTTTCGGC-3`
P2Y1	5`-TGTTCAATTTGGCTCTGGC-3`	5`-AGATGAAATAACTTCGCAGG-3`
P2Y2	5`-CTTCGCCCTCTGCTTCCTG-3`	5`-TTGGCATCTCGGGCAAAGC-3`
P2Y4	5`-GGCATTGTCAGACACCTTG-3`	5`-AAGACAGTCAGCACCACAG-3`
P2Y6	5`-CGCTTCCTCTTCTATGCCA-3`	5`-AGGCTGTCTTGGTGATGTG-3`
P2Y12	5`-GACTACAAGATCACCCAGG-3`	5`-CCTCCTGTTGGTGAGAATC-3`
P2Y13	5`-GCCGACTTGATAATGACAC-3`	5`-ATGATCTTGAGGAATCTGTC-3`
P2Y14	5`-TCTTTTACGTGCCCAGCTC-3`	5`-CTGTCAAAGCTGATGAGCC-3`
ENTPDase1	5`-AGCTGCCCCTTATGGAAGAT-3`	5`-TCAGTCCCACAGCAATCAAA-3`
ENTPDase2	5`-TTCCTGGGATGTCAGGTCTC-3	5`-GTCTCTGGTGCTTGCCTTTC-3`
ENTPDase3	5`-ACCTGTCCCGTGCTTAAATG`-3`	5`-AGACAGAGTGAAGCCCCTGA-3`
ENTPDase8	5`-CACACAGGACCTTCTGAGCA-3`	5`-AGCCTTCTGAGGTGGCACTA-3`
Ecto-5`-nucleotidase	5`-CAGGAAATCCACCTTCCAAA-3`	5`-AACCTTCAGGTAGCCCAGGT-3`
FIZZ1	5`-TCCCAGTGAATACTGATGAGA-3`	5`-CACTCTGGATCTCCCAAGA-3`
Ym1	5`-GGGCATACCTTTATCCTGAG-3`	5`-CCACTGAAGTCATCCATGTC- 3`
β-actin	5`-TATGCCAACACAGTGCTGTCTGG-3`	5`-TACTCCTGCTTGCTGATCCACAT-3`

TABLE 2. Expression of P1, P2 receptors and ectonucleotidases as determined by RT-PCR.

Genes	Resident	LPS	IL-4
P2rx1	-	-	-
P2rx2	-	-	-
P2rx3	-	-	-
P2rx4	+	+	+
P2rx5	-	-	-
P2rx6	-	-	-
P2rx7	+	+	+
P2ry1	+	+	+
P2ry2	+	+	+
P2ry4	-	-	-
P2ry6	±	±	±
P2ry12	±	±	±
P2ry13	-	-	-
P2ry14	+	+	+
Adora1 (A1)	±	±	±
Adora2a (A2A)	+	+	+
Adora2b (A2B)	±	±	±
Adora3 (A3)	+	+	+
Entpd1	+	+	+
Entpd2	-	-	-
Entpd3	+/-	+/-	+/-
Entpd8	-	-	-
Nt5e	+	+	+

The same pattern of expression was observed in all the experiments (n=5). +: strong expression; ±: barely detectable; –: no signal detected.

3.2 Artigo 2

HOMOCYSTEINE POLARIZES MACROPHAGES TO PRO-INFLAMATORY PHENOTYPE AND ALTERS E-NTPDase AND ECTO-5'-NUCLEOTIDASE ACTIVITIES

Rafael Fernandes Zanin, Letícia Scussel Bergamin, Elizandra Braganhol, Maria Martha Campos, Alfeu Zanotto Filho, José Cláudio Fonseca Moreira, Fermanda Morrone, Maria Rosa Chitolina Schentinger, Angela Terezinha de Souza Wyse and Ana Maria Oliveira Battastini.

Manuscrito a ser submetido para publicação

HOMOCYSTEINE POLARIZES MACROPHAGES TO PRO-INFLAMATORY PHENOTYPE AND ALTERS E-NTPDase AND ECTO-5'-NUCLEOTIDASE ACTIVITIES

Rafael Fernandes Zanin[1], Letícia Scussel Bergamin[1], Elizandra Braganhol[1], Maria Martha Campos[2], Alfeu Zanotto Filho[1], José Cláudio Fonseca Moreira[1], Fermanda Morrone[2], Maria Rosa Chitolina Schentinger[3], Angela Terezinha de Souza Wyse[1] and Ana Maria Oliveira Battastini[1]*.

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil

[2] Faculdade de Farmácia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brasil

[3]Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, RS, Brasil

***Corresponding author:**

Dra. Ana Maria Oliveira Battastini

Departamento de Bioquímica

Instituto de Ciências Básicas da Saúde

Universidade Federal do Rio Grande do Sul

Rua Ramiro Barcelos, 2600-anexo

Porto Alegre, RS, Brasil - 90035-003

E-mail: abattastini@gmail.com

ABSTRACT

Elevated plasma Hcy (hyperhomocysteinemia-HHcy) is independently associated with development of coronary artery disease (CAD), peripheral vascular disease and thrombosis. Recent studies have been shown that the inflammatory arm of the immune system is intimately linked to pathogenesis of atherosclerosis associated to HHcy. It has been hypothesized that macrophages play central roles in the initiation and in the progression of atherosclerosis. In the present study, we evaluated if elevated Hcy levels could alter macrophage phenotype. The aim of this study was to evaluate the effect of Hcy on nucleotide hydrolysis in macrophages by measuring NTPDase and ecto-5'-nucleotidase activities. The results demonstrated that micromolar concentrations of Hcy (50 μM and 100 μM) induced macrophages to the pro-inflammatory phenotype (M1). Moreover, Hcy increased E-NTPDase and ecto-5'-nucleotidase activities. The E-NTPDase3 presented a high protein level in macrophages treated with Hcy whereas E-NTPDase1 and ecto-5'-nucleotidase were unaltered. In conclusion, the macrophages exposed to Hcy present a polarized pro-inflammatory profile (M1) and our findings provide evidences of the involvement of the E-NTPDase3 and ecto-5'-nucleotidase in the inflammatory complications associates to HHcy

Keywords: Homocysteine, Macrophages, ecto-5'-nucleotidase, E-NTPDase

1. INTRODUCTION

Homocysteine (Hcy) is a sulphur-containing amino acid that is closely related to the amino acid methionine [1]. Raised plasma Hcy (hyperhomocysteinemia- HHcy) is independently associated with development of coronary artery disease (CAD), cerebral and peripheral vascular disease and thrombosis. [2, 3, 4]. The underlying mechanisms of how Hcy contributes to the pathogenesis of atherosclerosis are still poorly understood. Recent studies have been shown that the inflammatory arm of the immune system is intimately linked to pathogenesis of atherosclerosis [5, 6, 7]. Likewise, in patients with cardiovascular disease, neurodegenerative and autoimmune disorders, rather close associations have been described between the concentrations of total Hcy and inflammation markers: for example, neopterin [8, 9] and C-reactive protein [10]. Moreover, stimulatory effects in the production of pro-inflammatory cytokines have also been associated to the elevated plasma level of Hcy, including: IL-6, IL-12, IL-18 and IL-1β [11, 12, 13, 14, 15]. Therefore, Hcy in increased concentrations, by metabolic and/or nutrition conditions, might act as a pro-inflammatory molecule and modulate immune functions (ref).

Macrophages are present in the initial atherosclerotic lesion and has been hypothesized that it plays a central role in the progression of the atherosclerosis. Macrophages are key cells in the inflammatory process, and are characterized by a marked phenotypic heterogeneity depending on their microenvironmental stimulation [16, 17, 18] Different phenotypes may exist at various stages of disease progression, as for example in atherosclerosis diseases. Classical activation of macrophages (M1) is

characterized by production of pro-inflammatory cytokines, phagocytoses and kills of invading microorganisms, and initiates the adaptive immune responses [19, 20] whereas, alternative activation of macrophages (M2) coordinate the repairing process following an inflammatory reaction. These functional differences are reflected in the expression levels of surface proteins, mRNA target genes, the inflammatory cytokine production and the profile of two opposing effector molecules, inducible nitric oxide synthase (iNOS) and arginase (ref).

Extracellular nucleotide ATP and its breakdown products, as adenosine (Ado), function as endogenous signaling molecules that control inflammation and immune responses [19]. ATP acts as a pro-inflammatory agent, mainly through the control of secretion of cytokines, such as IL-1β, IL-6 and TNF-α whereas Ado acts as antiinflammatory agent diminishing. TNF-α secretion and increased the IL-10 production [21]. A large family of membrane-bound receptors identified as P2 and P1 mediates cell signaling by nucleotides and nucleosides, which are expressed on immune cells [22, 23]. The P2 family is divided in two subfamily: the ion-channel $P2X_{1,2,3,4,5,6,7}$ receptors and the G-protein-coupled $P2Y_{1,2,4,6,11–14}$ receptors [22]. The P1 are composed of four members A_1, A_{2A}, A_{2B} and A_3 that bind extracellular Ado [22].

The nucleotide/nucleoside levels in the extracellular medium are predominantly lowest but pathological events, such as cell death and inflammation, can increase dramatically their levels. Enzymes catalyzing their conversion control the concentration of nucleotides/nucleosides in the extracellular compartment. The combined family of enzymes are known as ectonucleotidases. Most prominent of these enzyme families are ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) and ecto-5'-

nucleotidase/CD73 (ecto-5'-NT/CD73) [24, 25]. The E-NTPDases efficiently hydrolyze nucleoside 5`-triphosphates and –diphosphates (physiological ATP, UTP, ADP and UDP) to its respective nucleoside 5'-monophosphate. The NTPDase family comprises eight members and among these, four present plasmatic membrane bound forms: E-NTPDase1/CD39, E-NTPDase2, NTPDase3 and E-NTPDase8, which differ in the preference for substrates. The AMP is hydrolyzed by ecto-5′-nucleotidase/CD73 (ecto-5′-NT/CD73) with subsequent release of adenosine in the extracellular space [24, 25]. The E-NTPDase1/CD39 and ecto-5′-nucleotidase/CD73 are distributed on the membrane of different blood cells and are involved in the inhibition of platelet recruitment and thrombus formation [26] leukocyte migration [27] and immunessupressive functions [28].

In the present study, we evaluated if elevated Hcy levels could alter macrophage phenotype and, at the same time, we investigated the effect of Hcy on the activity of the enzyme involved in nucleotide hydrolysis in murine macrophages.

2. MATERIALS AND METHODS

2.1 Animals and Reagents

CF-1 male mice, 6 - 8 weeks old, were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.), at a room temperature of 22 ± 2°C. The mice had free access to standard laboratory mice chow and water. The animal handling and experiments were performed in accordance with the international guidelines

in compliance with the Federation of Brazilian Societies for Experimental Biology. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Macrophages preparation and Hcy treatment

Peritoneal macrophages were collected by a lavage of the peritoneal cavity with 5 mL of sterile RPMI-1640 medium without fetal bovine serum (FBS). The cells were washed twice with sterile Phosphate Buffered Saline (PBS) and suspended in RPMI without FBS. The cells obtained were transferred to 6, 24 or 48 multiwell plates and allowed to attach for 30 minutes. Unattached cells were washed out with RPMI without FBS. The attached cells, mainly peritoneal macrophages, were used for the experiments thereafter. Macrophage was evaluated by microscopic examination of the cultures after May-Grrunwald and Giemsa stains, indicating macrophage purity higher than 80%, which were confirmed with CD11b Ab.

For test the effect of Hcy, macrophages were treated 30 minutes after the attachment for 24h with 50 μM and 100 μM L, D homocysteine (Sigma Chemical Co.) in complete medium (RPMI 10% FBS). These concentrations of 50 μM and 100 μM correspond to 25 μM and 50 μM L-homocysteine, respectively. Theses concentrations can be found in hyperhomocisteinemic individuals [29].

2.3 Arginase and Nitrite Assay

Arginase activity in cell lysates was measured based on the conversion of L-arginine to L-ornithine and urea according to the technique described by Corraliza and collaborates [30] with minor modifications. Briefly, cells were lysed for 30 min with 40 µL of 0,1% Triton-X-100. Thirty µL of 25 mM Tris-HCl, pH 7.4 and 10 µL of 10 mM $MnCl_2$ were added, and the enzyme was heat-activated for 10 min at 56 ^{o}C. Similar amounts of sample (40 µL) and 0.5 M L-arginine (pH 9.7) were mixed and incubated for 1 hr at 37 ^{o}C. The reaction was stopped with 400 µL of H_2SO_4 (96%), H_3PO_4 (85%), H_2O (1/3/7, v/v/v). The urea concentration was measured at 540 nm after the addition of 8 µL of α-isonitropropiophenone 6%, followed by heating at 95^{o}C for 30 min. Values were compared with a standard curve of urea concentration.

Nitrite concentrations were measured using the Greiss reaction [31]. In brief, 200 µL of the tested supernatant were incubated with 100 µL of 1 % sulfanilamide and 100 µL of 0.3% N-1-naphthylethylenediamine dihydrochloride at room temperature for 5 min. Nitrite was quantified by spectrophotometry at 540 nm using sodium nitrite as a standard.

2.4 Determination of cytokine release

Supernatants were harvested ands the levels of tumor necrosis factor (TNF-α; R & D Systems) and IL-10 (R & D Systems) were determined by enzyme-linked immunoabsorbent assay (ELISA), according to the manufacturer's instructions.

2.5 Ectonucleotidase assay

To determine the ATPase, ADPase and AMPase activities, the 48 multiwell plates containing macrophages cells were washed three times with incubation medium in absence of nucleotides. The enzymatic reaction was started by the addition of 200 μL of incubation medium containing 2 mM $CaCl_2$ (2 mM of $MgCl_2$ to AMPase assay), 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4 and 2 mM of ATP, ADP or AMP as substrates, at 37^{o}C. After 10 min of incubation the reaction was stopped by collecting an aliquot of the incubation medium and transferring it to eppendorf tubes containing trichloroacetic acid (final concentration 5% w/v), previously placed on ice. The release of inorganic phosphate (Pi) was measured by the malachite green method [32], using KH_2PO_4 as a Pi standard. The non-enzymatic Pi released from nucleotides into the assay medium without cells was subtracted from the total Pi released during the incubation, giving net values for enzymatic activity. All samples were run in triplicate. Specific activity was expressed as nmol Pi released/min/mg of protein. The protein concentration was measured by the Coomassie Blue method using bovine serum albumin as standard [33]. For antioxidant test Trolox, a water-soluble analog of vitamin E, was add 30 minutes before to Hcy (50 μM) in culture of macrophages.

2.6 HPLC

HPLC analysis was used to determine and quantify the nucleotide products of ATP and AMP hydrolysis. The cells were incubated as described above, except that ATP or AMP concentrations were 100 μM. To stop the reaction, an aliquot of the incubation

medium was transferred to an eppendorf tube on ice and centrifuged at 4 ^{0}C for 30 min at 16,000 g. Aliquots of 40 µL were applied to a reverse phase HPLC system using a C18 Shimadzu column (Shimadzu, Japan) with absorbance measured at 260 nm. The mobile phase was 60 mM KH_2PO_4, 5 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol as described [34]. Retention times were assessed using standard samples of nucleotide and their metabolites. The non-enzymatic hydrolysis of the ATP and AMP were consistently less than 5% and cells incubated without the addition of nucleotides did not present any detectable peak.

2.7 RT-PCR and Real Time PCR

The RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, California, USA). Approximately 1 µg of total RNA were added to each cDNA synthesis reaction using the SuperScript-III RT pre-amplification system (Invitrogen). The PCR reactions were performed in 25 µL of the reaction mixture

containing 1 µL cDNA, 10 pmol of the primer in the supplied PCR mix buffer (Platinum PCR Supermix, Invitrogen). After initial denaturation for 5 min at 95°C, the amplifications were carried out for 35 cycles of denaturation at 94°C for 45 s, annealing at primer specific temperature for 45 s and extension at 72°C for 45 s. The PCR was ended by 7 min incubation at 72°C. The same program was used for the amplification of the gene of reference, which was β-actin. Sequences of primers are listed in Table 1. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized with SYBRGolg (Molecular Probe).

Real-time PCRs were carried out in the Applied-Biosystem Step One Plus cycler using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) following the manufacturer's instructions and performed in triplicate. Total RNA and cDNA were generated, as described in RT-PCR analysis. The selected primers used for real time PCRs were the same used in RT-PCR analysis. All results were analyzed by the $2^{-\Delta/\Delta CT}$ method [35]. The β-actin was used as the internal control gene for all relative expression calculations.

2.8 Flow Cytometry

The expression of selected surface proteins by peritoneal macrophages was evaluated by cytometry using the following antibodies (Ab): guinea pig polyclonal anti-mouse NTPDase1/CD39 (mN1-1_c (I_4, I_5)), rabbit polyclonal anti-mouse NTPDase2 (mN2-36L), guinea pig anti-mouse NTPDase3 (mN3-1_cI_4) and rabbit anti-rat ecto-5`-nucleotidase/CD73 (rNu 9_L, $I_{4,}5$). Briefly, the cells were incubated for 30 min with the above primary Ab diluted in PBS, 1% FBS, 0.1% sodium azide (PFA), and, when necessary, with secondary FITC-conjugated goat anti-rabbit IgG Ab (Invitrogen) or Alexa 488-conjugated goat anti-guinea pig IgG Ab (Invitrogen) for 30 min, with a minimum of two washes with PFA after each incubation. Cell surface fluorescence was measured with FACSCalibur Flow Cytometer (BD Biosciences).

2.9 Statistical analysis

Data were expressed as mean ± S.D. and were subjected to one-way analysis of variance (ANOVA) followed by Tukey post-hoc test (for multiple comparisons). Differences between mean values were considered significant when $P<0.05$.

3. RESULTS

3.1 Effect of Hcy on macrophage activation

To define the effect that Hcy confers in the context of macrophages activation, we examined arginase activity and nitrite production (iNOS activity), two markers of macrophage activation. It was observed that the treatment of macrophages with 50 and 100 μM Hcy produced a significantly increase in nitrite production in relation to the resident macrophages (Figure 1A) while these treatments did not affect the arginase activity (Figure 1B).Considering that Hcy has been shown to increase the production of pro-inflammatory cytokines both *in vivo* and *in vitro* [11, 12, 13, 14], we also evaluated the production of TNF-α and IL-10 by macrophages treated by the two concentrations of Hyc. The results demonstrated that macrophages treated with Hcy present an increased production of TNF-α when compared to the resident cells (Figure 1C, 1D). In addition low levels of IL-10 were secreted by macrophages exposed to both concentrations of Hcy.

In addition, to observe the type of macrophage activation promoted by Hcy, it was analyzed the Ym1 and FIZZ1 mRNA expression, which are strongly induced in alternative activated macrophages (M2) [36, 37, 18]. As it is showed in figure 1E, the treatment with Hcy did not alter the mRNA expression of these two markers.

Taking together, this set of results demonstrated that high levels of Hcy induced the macrophages to the pro-inflammatory phenotype (M1).

3.2 ATPase, ADPase and AMPase activities on macrophages exposed to Hcy

The ATPase and ADPase activities in macrophages treated with Hcy was significantly increased by 90% and 60% for ATP and ADP hydrolysis, respectively (Figure 2A) and it was no observed difference between the concentrations tested of Hcy (50 and 100 μM). The AMP hydrolysis was also significantly increased (~ 80%) in macrophages treated in both concentration of Hcy (50 and 100 μM) when compared to the resident cells . Here also was not verified difference in the AMP hydrolysis between Hcy concentrations (Figure 2B). Considering that there was no significant difference in the ATPDase and AMPase activities between the concentrations tested (50 and 100 μM), in the subsequent experiments it was not evaluated the 100 μM concentration.

The results showed in the figure 2, was confirmed by HPLC analysis. Figure 3 shows that ATP is degraded faster by macrophages exposed to Hcy in relation to resident cells. It is also possible to note the AMP is totally degraded in 60 min in macrophages treated with Hcy while in resident cells this occur at the end of 120 min, corroborated with the higher AMPase activity in cells that received Hcy. The other striking difference observed

was lower concentration of inosine, the final product of nucleotide metabolism, at the end of incubation (120 min) in Hcy treated macrophages (Figure 3 A, B).

3.4 Effects of the Hcy on ATPase, ADPase and AMPase activities with pre-treatment of trolox, a free radical scavenger

Here, we showed that Trolox did not prevent the enhancement in the ATPase and ADPase activities (Figure 4) but rather it was able to prevent the increasing promoted by Hcy treatment on ecto-5'-nucleotidase. This result suggest that generation of free radicals by Hcy may be involved in the modulation of the ecto-5'-nucleotidase activity.

3.3 Effects of Hcy one ectonucleotidases expression

To evaluate the P1, P2 and E-NTPDases (1, 2, 3 and 8) and ecto-5'-nucleotidase mRNA expression by the macrophages after treatment with Hcy, mRNA was isolated from cell cultures and it was analyzed by RT-PCR. The results showed that, the cells treated with Hcy did not present any difference in the mRNA expression of P1, P2 receptors and ectonucleotidases analyzed in comparison to resident macrophages (Table 2). The Real Time experiments demonstrated that E-NTPDase1, 3 and ecto-5'-nucleotidase gene expression was not significant in macrophages treated with Hcy when compared to the control (Figure 5).

Considering that the enzyme activity was altered by Hcy treatment, we aimed to determine whether E-NTPDases and ecto-5'-nucleotidase protein expression by flow

cytometry analysis. As shown in (figure 6A, B), Hcy treatment increased only the E-NTPDase3 protein level when compared to the resident macrophages.

4. DISCUSSION

One of the important features in the development of atherosclerosis is macrophages accumulation in the lesion. In the present study, we report that the Hcy (50 and 100 μM) polarize macrophages cells towards an M1 phenotype. This polarization is defined by FIZZ1 and Ym1 mRNA expression, cytokine production and arginase/iNOS activities. Consistent with previous reports, [38, 39, 13] our data demonstrate that Hcy has potent pro-inflammatory properties. The Hcy increased pro-inflammatory TNF-α and did not increase of anti-inflammatory IL-10 production. The pro-inflammatory phenotype of macrophages (M1) was also demonstrated by the fact that Hcy increased the production of nitrite (iNOS activity) while did not affect arginase activity. Finally, Hcy did not alter the Ym1 and FIZZ1 mRNA expression, which are strong markers of alternative activation (M2). This polarized pro-inflammatory macrophage phenotype may contribute to development of atherosclerosis related to pathological concentrations of Hcy.

It is well known that ATP and its breakdown product adenosine play important role in inflammatory process including macrophages effectors action [20]. Our results also demonstrated that exposition of macrophages to Hcy increased ATP hydrolysis when compared to resident cells; the results also revealed that there was no difference in these effects regarding the Hcy concentration (50 μM or 100 μM). The HPLC measurements showed that ATP degradation is faster in macrophages treated with Hcy than in resident

cells. On the other hand, the Real Time-PCR fail in demonstrate differences at mRNA levels of the E-NTPDases1 and E-NTPDase3 in the macrophages exposed to Hcy in relation to resident cells. In addition, Hcy was also not able to alter protein level expression of E-NTPDase1 but it was observed a significant increase in the protein level of E-NTPDase3 when compared to the resident cells. The increasing in the ATPase:ADPase ratio when the cells were treated with Hcy (figure 2A) led us to suggest that the pivotal ecto-enzyme altered in macrophages treated with Hcy is E-NTPDase3, as supported by increased in the protein level of E-NTPDase3 (figure 6).

Another important finding is that the treatment with Hcy concentration caused an increasing of approximately 80% in the AMP hydrolysis when compared to resident cells. In fact, the HPLC analysis demonstrated that AMP was completely degraded in approximately 60 min in macrophages treated with Hcy while in resident cells it continued until 120 min. Interesting, the generation of inosine were diminished by the treatment with Hcy (Figure 3A, B). The increasing in the AMP hydrolysis observed was not related to mRNA transcript and protein distribution of the ecto-5'-nucleotidase (Figures 5, 6).

Our results demonstrated changes in the ATPase, ADPase and AMPase in the macrophages treated with Hcy. Considering that high levels of extracellular nucleotides are present in cellular damage/lysis, platelet degranulation, and at sites of inflammation, ectonucleotidases, such as E-NTPDase and ecto-5'-nucleotidase, play a crucial role in the control of levels of signaling molecules in the microenvironment of immune cells during inflammation [20, 25, 27]. Then, the rapid breakdown of ATP and ADP, induced by Hcy treatment simultaneously with the increase in the AMP hydrolysis might enhance the

complete enzymatic cascade that allow the hydrolysis of ATP to adenosine, that is classically known as a power antiinflammatory molecule on immune cells. However, the macrophages present pro-inflammatory characteristics, opposite to the action of the adenosine. A possible explanation for this apparently contradictory result is that during the process of methylation, *S*-adenosylmethionine (SAM) derived from methionine is converted to *S*-adenosylhomocysteine (SAH), which is additionally hydrolyzed to produce Hcy and adenosine by SAH hydrolase [40]. The reaction involving S-adenosylhomocysteine (AdoHcy) hydrolysis to form homocysteine and adenosine is reversible [41]. Although the equilibrium constant of this reaction favors AdoHcy synthesis, under physiological conditions AdoHcy is hydrolyzed to homocysteine and adenosine. In hyperhomocysteinemia, the reaction shifts toward AdoHcy synthesis at the expense of free intracellular adenosine. In this aspect, previous studies demonstrated that elevated concentration of Hcy on plasma or tissue induced fall in extracellular adenosine via dipyridamole sensitive equilibrative nucleoside transporter [42, 43]. Therefore, our results indicate that the decrease in the level of inosine generation in macrophages treated with Hcy could be related to the increase in the adenosine uptake from the extracellular microenvironment, impairing the adenosine action via adenosine receptors. Thus, it can be speculated that adenosine generated by the rapid ATP, ADP and AMP hydrolysis in macrophages treated with Hcy is uptake into the cell, what could maintain a pro-inflammatory activated status and contributing to the inflammatory complications in the atherosclerotic plaque associated to the hyperhomocysteinemia. Additionally, it was demonstrated here that Hcy induced an increase in TNF-α levels and nitrite production that drive the macrophage to a pro-inflammatory phenotype.

Potential mechanisms have been implicated in the pathology of hyperhomocystinemia that including formation of reactive oxygen species and Hcy or Hcy metabolites bound to protein (homocysteinylation) [44]. In this context, the Trolox, a radical scavenger, failed in demonstrate any involvement of oxidative stress related to Hcy effects on increase of the E-NTPDase activity when macrophages received pre-treatment with Trolox. Nevertheless the addition of trolox, partially prevented the increase of the ecto-5'-nucleotidase activity by Hcy exposition of macrophages. This result may indicate that free radical formation is involved on Hcy effects related to the enhancement in the ecto-5'-nucleotidase activity (figure 4).

In conclusion, the results presented here demonstrate that macrophages exposed to Hcy showed a polarized profile pro-inflammatory (M1) and an increase in nucleotide hydrolysis, which provide evidences of the involvement of the E-NTPDase3 and ecto-5'-nucleotidase in the inflammatory complications associates to HHcy in macrophages.

5. ACKNOWLEGEMENTS

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7. LEGENDS

Figure 1. Macrophages treated with Hcy exhibited different patterns of arginase/iNOS activities (A), mRNA expression (B) and cytokines profile (C). (A) NO^{2-} (nitrite) accumulation (iNOS activity) in macrophages after 24h of treatment with Hcy (50 μM and 100 μM). Arginase assay measuring the formation of urea after incubation of lysates from activated macrophages with arginine. Data show mean±SD of 5 independent experiments. (*) $p<0.001$, two-way ANOVA with Tukey's post-hoc test. (B) TNF-α and IL-10 cytokines were measured from supernatants of macrophages treated with Hcy and resident cells. Concentrations are depicted as mean+SD and data are representative of three triplicate experiments. $p<0.01$, two-way ANOVA with Tukey's post-hoc test. (C) Effect of Hcy on FIZZ1 and Ym1 expression in macrophages were quantified by qPCR. The total the amount of FIZZ1 and Ym1 mRNA was normalized to β-actin signals and expressed as $2^{-\Delta/\Delta CT}$. Data show mean±SD for qPCR performed in 3 independent experiments with RNA purified from macrophages obtained from 6 to 8 individual mice carried out separately.

Figure 2. (A) ATP, ADP and (B) AMP hydrolysis in macrophages treated with Hcy (50 μM and 100 μM). The data represent mean ± S.D. (n=6) with pooled macrophages from 6 to 8 mice per experiment carried out separately. Data were performed by ANOVA, followed by Tukey's test. (*) Represent significant statistical difference compared to the resident macrophages, considering $P<0.05$ as significant.

Figure 3. Metabolism of extracellular ATP in macrophages treated with Hcy (50μM and 100 μM). Resident macrophages (A) and macrophages treated with Hcy 50μM (B) were incubated in 48 well plates 100 μM of ATP in 200 ml of incubation medium as described in material and methods. An aliquot of the supernatant was withdrawn at 0, 10, 20, 40, 60 and 120 min and the presence of ATP, ADP, AMP, Adenosine (ADO) and inosine (INO) were determined after separation by HPLC. Data are mean±SD (bars) values from three experiments in triplicates.

Figure 4. The effects of Trolox addition on (A) ATP, ADP and (B) AMP hydrolysis in macrophages treated with Hcy (50μM). The data represent mean ± S.D. (n=3) with pooled macrophages from 3 mice per experiment carried out separately. 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 5. E-NTPDase1, E-NTPDase3 and ecto-5`-nucleotidase expression was quantified by qPCR. The total the amount mRNA were normalized to β-actin signals and expressed as $2^{-\Delta/\Delta CT}$. Data show mean±SD for real time PCR experiments performed in triplicate with RNA purified from three independent experiments with pooled macrophages from 6 to 8 mice per experiment. Macrophages treated with Hcy (50μM) were compared to resident cells (*) $p<0.001$, two-way ANOVA with Tukey's post-hoc test.

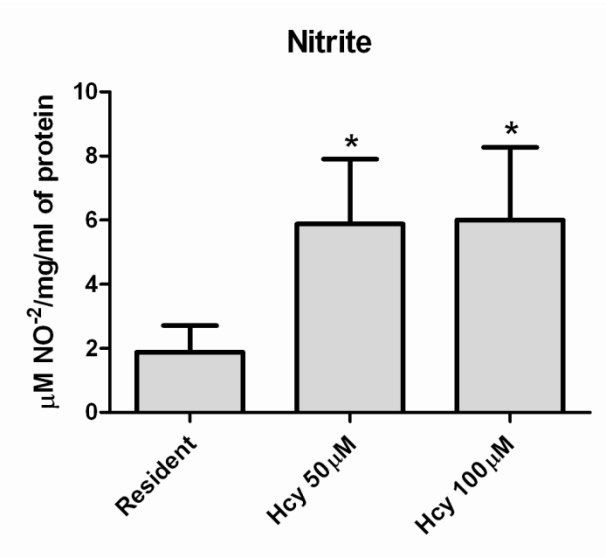
Figure 6. Flow cytometry profiles for (A) E-NTPDase1, E-NTPDase3 and (B) ecto-5`-nucleotidase on macrophages 24 h after Hcy treatment. Changes in expression are

assessed by comparison against resident macrophages. Figures are representative of at least three independent experiments with pooled macrophages from 6 mice per experiment.

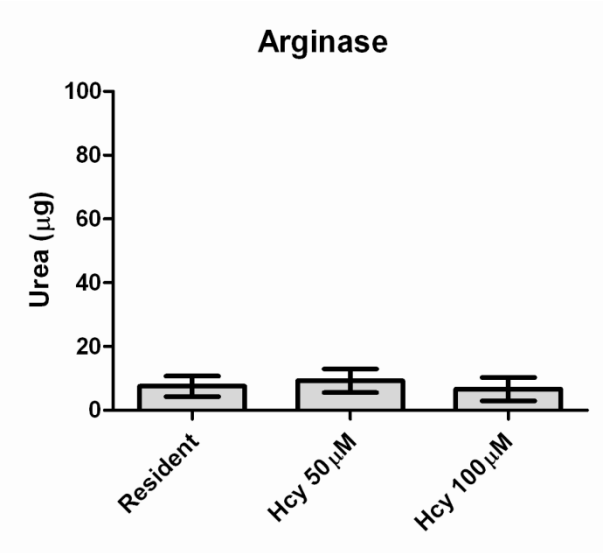
8. FIGURES

FIGURE 1

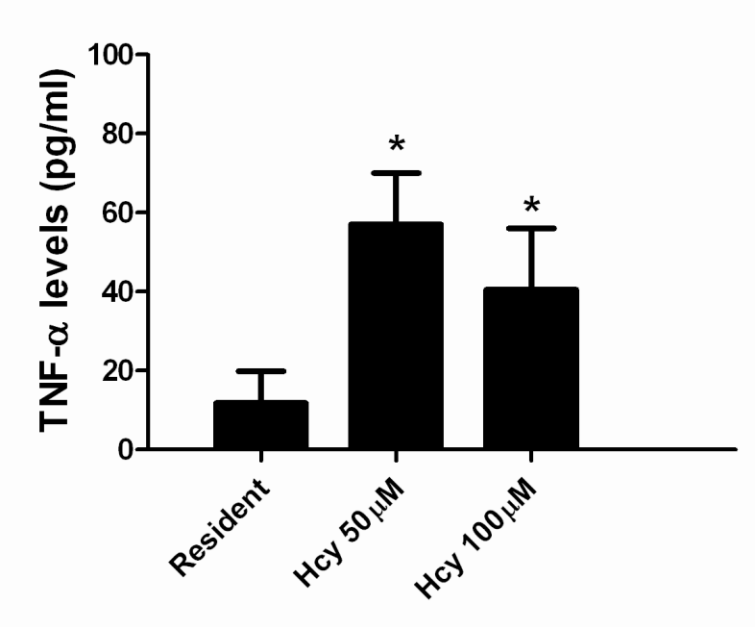
(A)



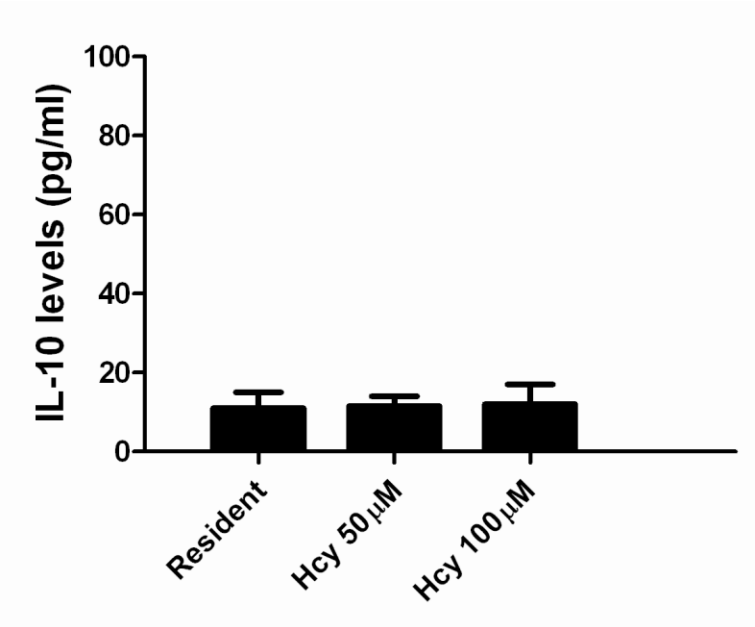
(B)



(C)


TNF-α levels (pg/ml)
100
80
60
40
20
0
*
*
Resident
Hcy 50μM
Hcy 100μM

(D)


IL-10 levels (pg/ml)
100
80
60
40
20
0
Resident
Hcy 50μM
Hcy 100μM

(E)

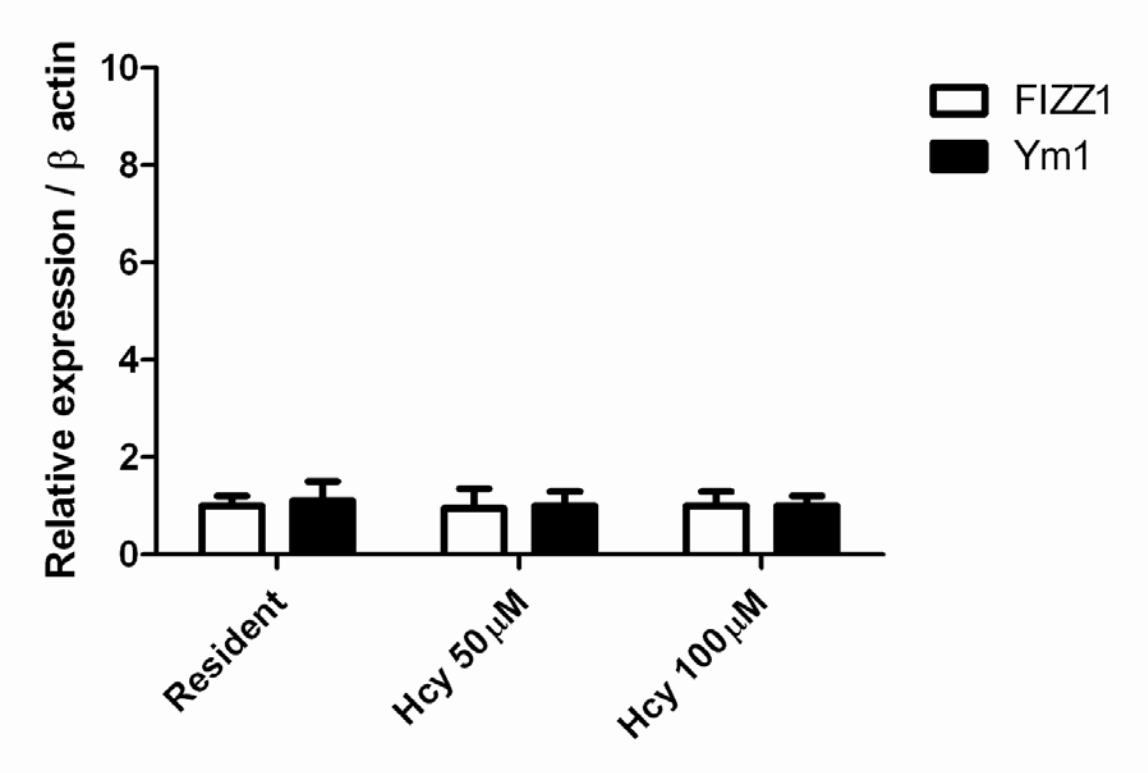
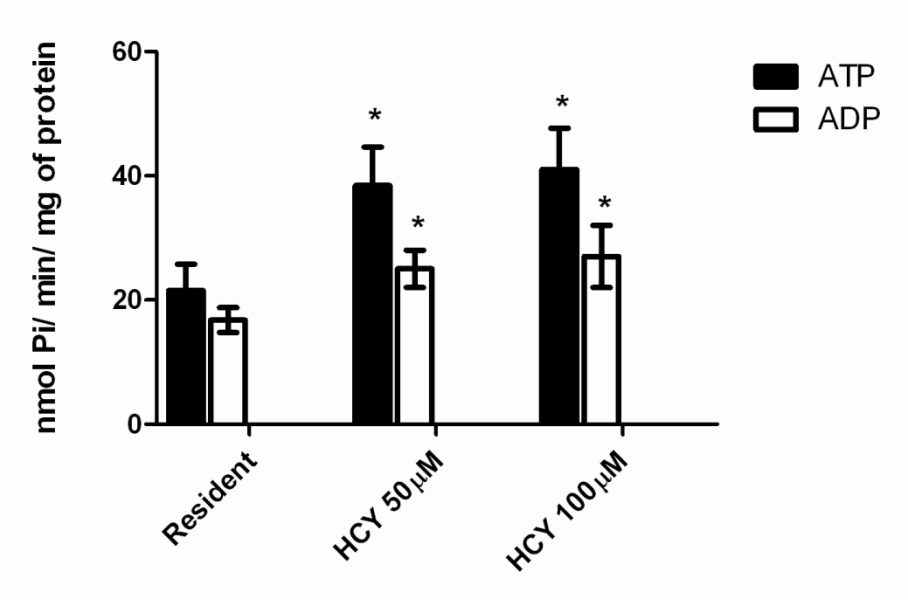

Relative expression / β actin
10
8
6
4
2
0
FIZZ1
Ym1
Resident
Hcy 50μM
Hcy 100μM

FIGURE 2

(A)


nmol Pi/ min/ mg of protein
60
40
20
0
ATP
ADP
*
*
*
*
Resident
HCY 50μM
HCY 100μM

(B)

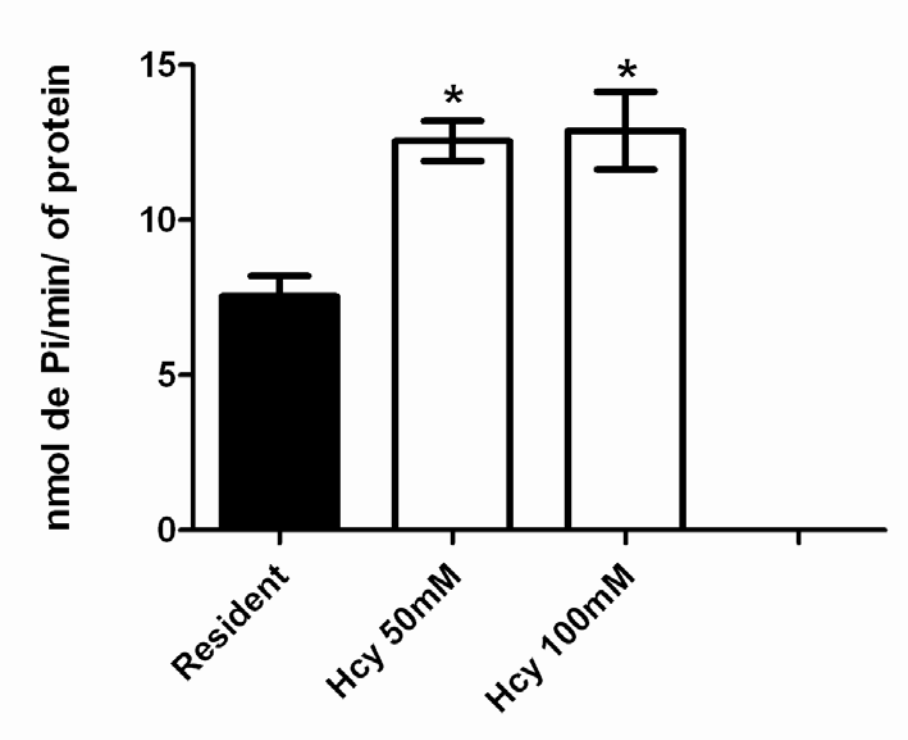
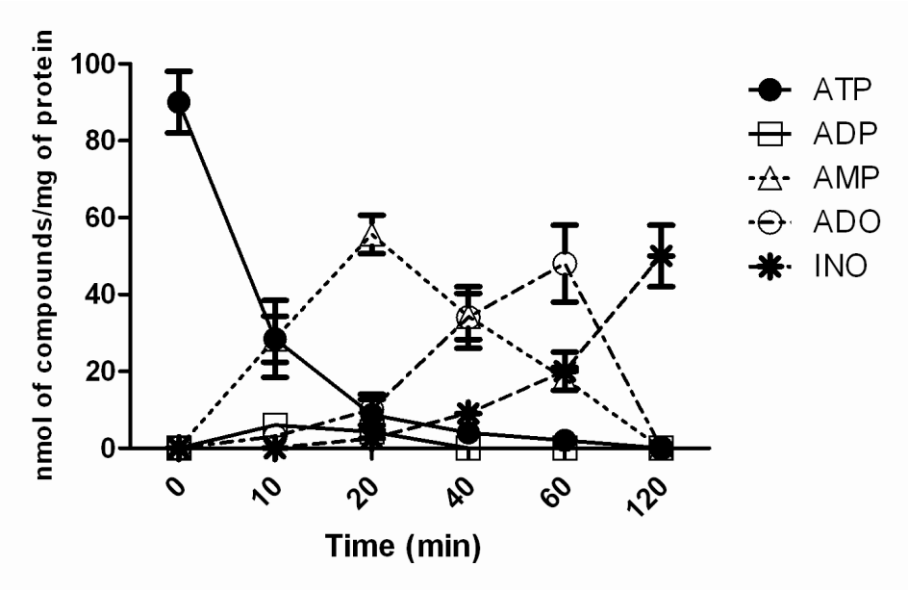

nmol de Pi/min/ of protein
15
10
5
0
*
*
Resident
Hcy 50mM
Hcy 100mM

FIGURE 3

(A)


nmol of compounds/mg of protein
100
80
60
40
20
0
0
10
20
40
60
120
Time (min)
ATP
ADP
AMP
ADO
INO

(B)

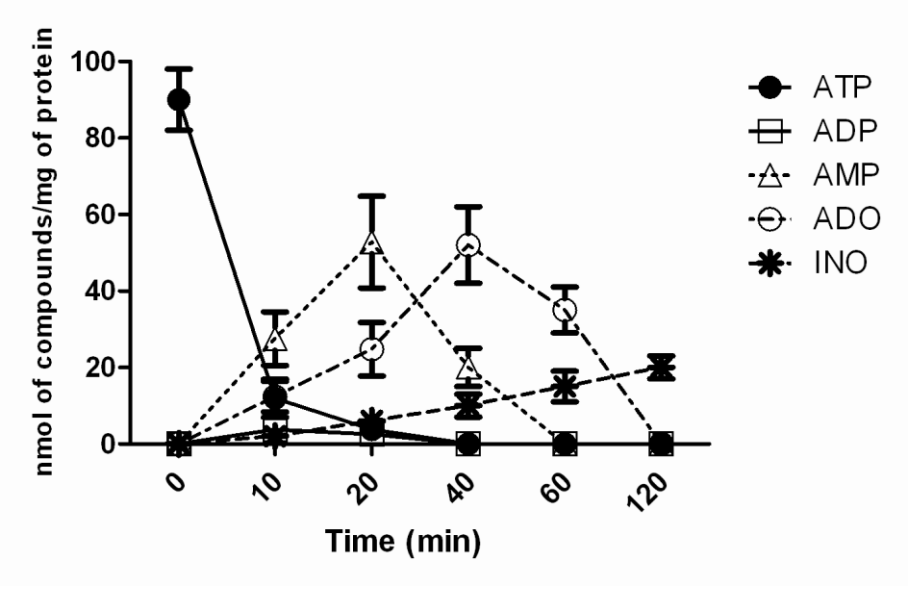

nmol of compounds/mg of protein
100
80
60
40
20
0
0
10
20
40
60
120
Time (min)
ATP
ADP
AMP
ADO
INO

FIGURE 4

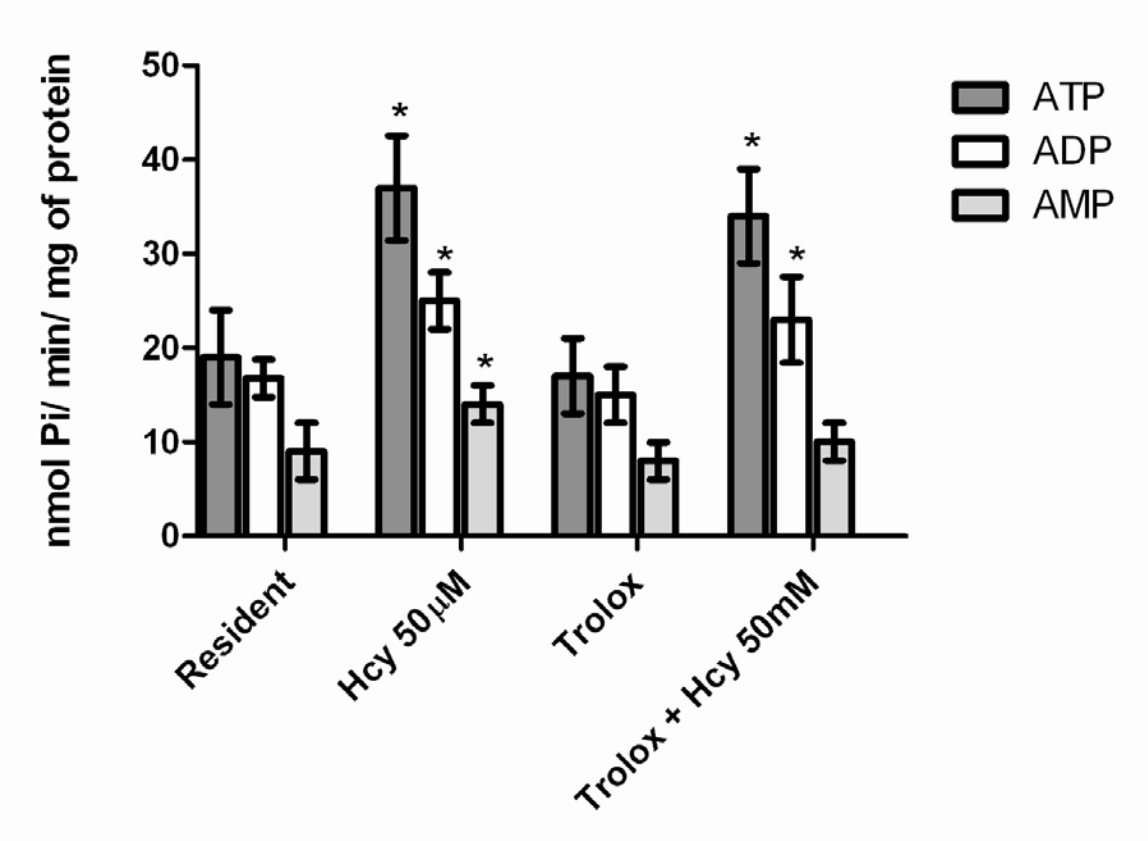

nmol Pi/ min/ mg of protein
50
40
30
20
10
0
*
*
*
*
*
ATP
ADP
AMP
Resident
Hcy 50μM
Trolox
Trolox + Hcy 50mM

FIGURE 5

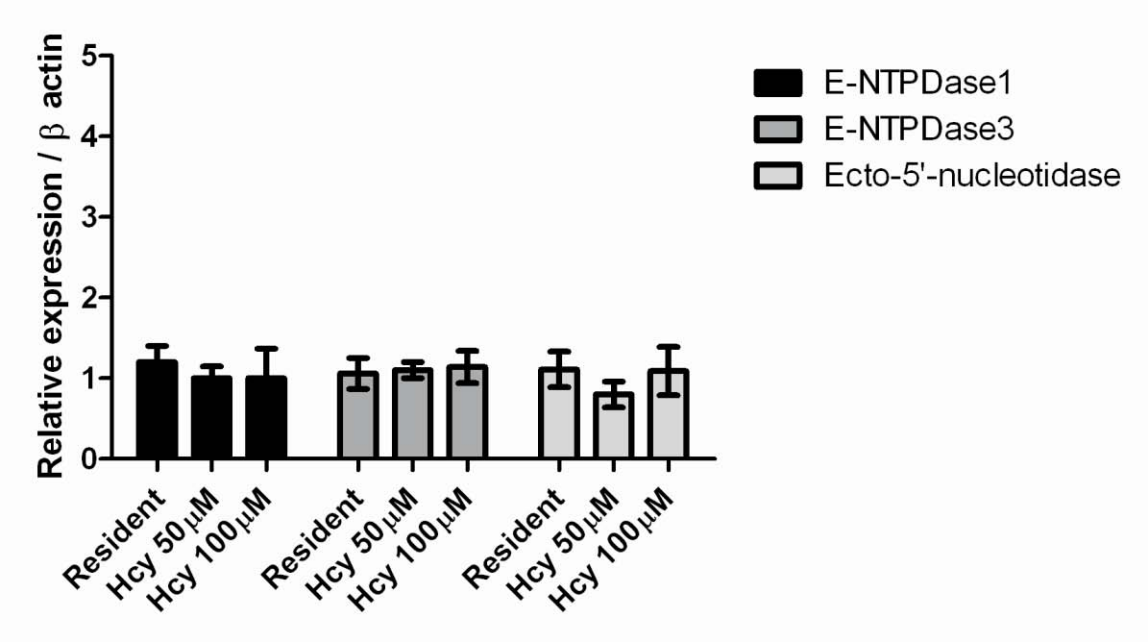
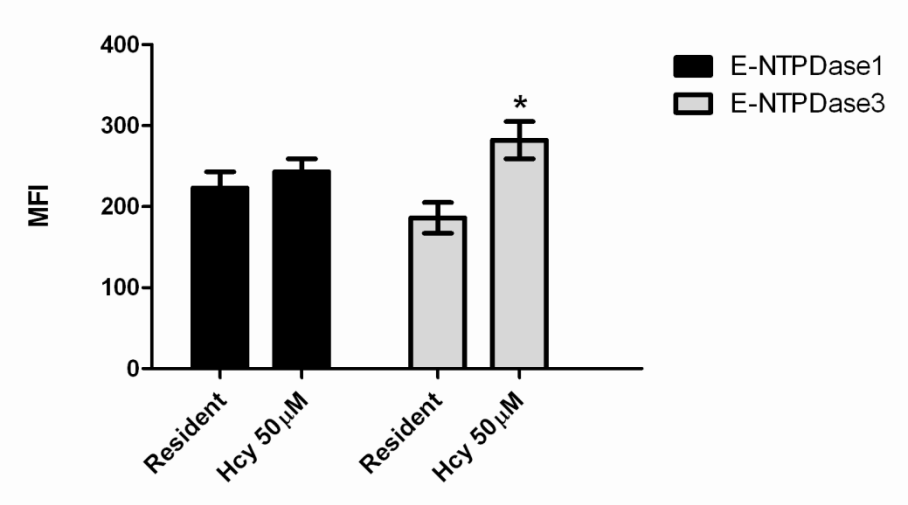

Relative expression / β actin
5
4
3
2
1
0
E-NTPDase1
E-NTPDase3
Ecto-5'-nucleotidase
Resident
Hcy 50μM
Hcy 100μM
Resident
Hcy 50μM
Hcy 100μM
Resident
Hcy 50μM
Hcy 100μM

FIGURE 6

(A)


400
300
200
100
0
MFI
*
E-NTPDase1
E-NTPDase3
Resident
Hcy 50 μM
Resident
Hcy 50 μM

(B)

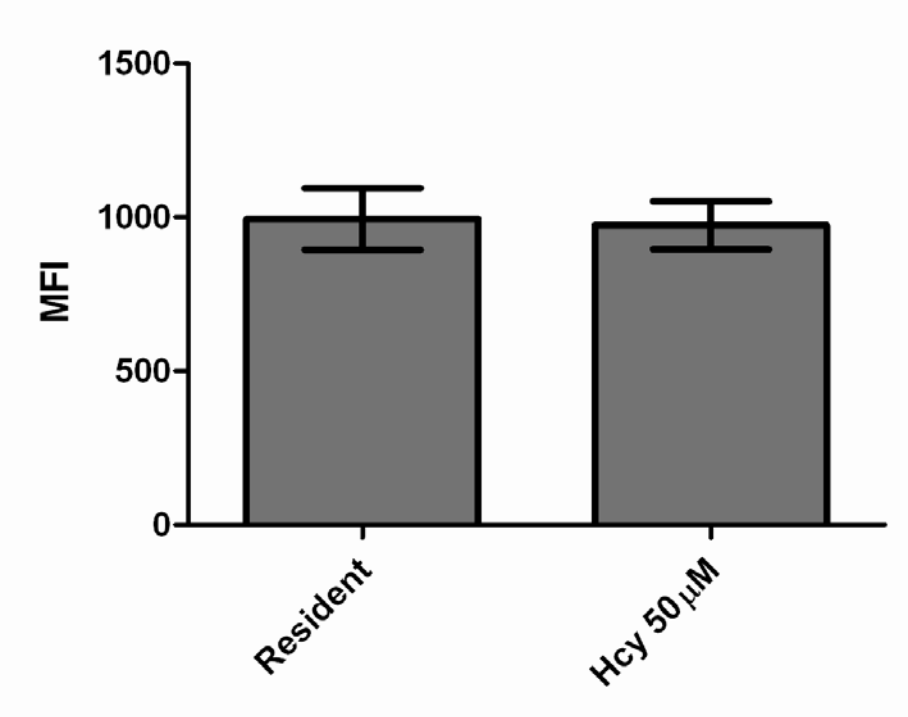

1500
1000
500
0
MFI
Resident
Hcy 50 μM

TABLE 1. Primers for purinergic receptors and ectonucleotidases used at RT-PCR and real time PCR Experiments.

Primer	Sense	Antisense
P2X1	5`-AAGGTCAACAGGCGCAACC-3`	5`-AACACCTTGAAGAGGTGACG-3`
P2X2	5`-GTGCAGAAAAGCTACCAGG-3`	5`-GGATGGTGAAATTTGGGGC-3`
P2X3	5`-GCTGCGTGAACTACAGCTC-3`	5`-ACTGGTCCCAGGCCTTGTC-3`
P2X4	5`-CTTGGATTCCGGATCTGGG-3`	5`-GGAATATGGGGCAGAAGGG-3`
P2X5	5`-GCACCTGTGAGATCTTTGC-3`	5`-TCGGAAGATGGGGCAGTAG-3`
P2X6	5`-CAGGACCTGTGAGATCTGG-3`	5`-TCCTGCAGCTGGAAGGAGT-3`
P2X7	5`-TCCCTTTGCAGGGGAACTC-3`	5`-GTACGGTGAAGTTTTCGGC-3`
P2Y1	5`-TGTTCAATTTGGCTCTGGC-3`	5`-AGATGAAATAACTTCGCAGG-3`
P2Y2	5`-CTTCGCCCTCTGCTTCCTG-3`	5`-TTGGCATCTCGGGCAAAGC-3`
P2Y4	5`-GGCATTGTCAGACACCTTG-3`	5`-AAGACAGTCAGCACCACAG-3`
P2Y6	5`-CGCTTCCTCTTCTATGCCA-3`	5`-AGGCTGTCTTGGTGATGTG-3`
P2Y12	5`-GACTACAAGATCACCCAGG-3`	5`-CCTCCTGTTGGTGAGAATC-3`
P2Y13	5`-GCCGACTTGATAATGACAC-3`	5`-ATGATCTTGAGGAATCTGTC-3`
P2Y14	5`-TCTTTTACGTGCCCAGCTC-3`	5`-CTGTCAAAGCTGATGAGCC-3`
ENTPDase1	5`-AGCTGCCCCTTATGGAAGAT-3`	5`-TCAGTCCCACAGCAATCAAA-3`
ENTPDase2	5`-TTCCTGGGATGTCAGGTCTC-3	5`-GTCTCTGGTGCTTGCCTTTC-3`
ENTPDase3	5`-ACCTGTCCCGTGCTTAAATG`-3`	5`-AGACAGAGTGAAGCCCCTGA-3`
ENTPDase8	5`-CACACAGGACCTTCTGAGCA-3`	5`-AGCCTTCTGAGGTGGCACTA-3`
Ecto-5`-nucleotidase	5`-CAGGAAATCCACCTTCCAAA-3`	5`-AACCTTCAGGTAGCCCAGGT-3`
FIZZ1	5`-TCCCAGTGAATACTGATGAGA-3`	5`-CACTCTGGATCTCCCAAGA-3`
Ym1	5`-GGGCATACCTTTATCCTGAG-3`	5`-CCACTGAAGTCATCCATGTC-3`
β-actin	5`-TATGCCAACACAGTGCTGTCTGG-3`	5`-TACTCCTGCTTGCTGATCCACAT-3`

TABLE 2. Expression of P2, P1 receptors and ectonucleotidases as determined by RT-PCR.

Genes	Resident	Hcy 50µM
P2rx1	-	-
P2rx2	-	-
P2rx3	-	-
P2rx4	+	+
P2rx5	-	-
P2rx6	-	-
P2rx7	+	+
P2ry1	+	+
P2ry2	+	+
P2ry4	-	-
P2ry6	±	±
P2ry12	±	±
P2ry13	-	-
P2ry14	+	+
Adora1 (A1)	±	±
Adora2a (A2A)	+	±
Adora2b (A2B)	±	±
Adora3 (A3)	+	±
Entpd1	+	+
Entpd2	-	-
Entpd3	+/-	+/-
Entpd8	-	-
Nt5e	+	+

The same pattern of expression was observed in all the experiments (n=5). +: strong expression; ±: barely detectable; –: no signal detected.

3.3 Artigo 3

HOMOCYSTEINE DECREASES EXTRACELLULAR NUCLEOTIDE HYDROLYSIS IN RAT PLATELETS

Rafael Fernandes Zanin, Luís Felipe Ingrassia Campesato, Elizandra Braganhol, Maria Rosa Chitolina Schetinger, Angela Terezinha de Souza Wyse, Ana Maria Oliveira Battastini

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Regular Article

Homocysteine decreases extracellular nucleotide hydrolysis in rat platelets

Rafael Fernandes Zanin [a], Luís Felipe Ingrassia Campesato [a], Elizandra Braganhol [a], Maria Rosa Chitolina Schetinger [b], Angela Terezinha de Souza Wyse [a], Ana Maria Oliveira Battastini [a,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, RS, Brazil

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ABSTRACT

Hyperhomocysteinemia is an independent risk factor for atherothrombotic disease. Platelets play an important role in cardiovascular disease and release pro-aggregates mediators when activated, such as ADP, a physiological agonist involved in normal hemostasis and thrombosis. NTPDases and 5'-nucleotidase are ecto-enzymes that hydrolyze ATP, ADP and AMP to adenosine playing an important role on blood flow and thrombogenesis by regulating ADP catabolism. The aim of the present study was evaluate extracellular adenine nucleotide hydrolysis of rat platelets exposed to homocysteine *in vitro* and *in vivo*. In vitro homocysteine (Hcy) in the concentration range of 20 to 500 μM caused a significant decrease on ATP (around 30%) and ADP (around 45%) hydrolysis, respectively, while AMP hydrolysis was not altered. Hcy was not able to inhibit the hydrolysis of ATP and ADP catalyzed by purified apyrase at the same concentrations tested *in vitro* on platelets, suggesting an indirect effect. The inhibitory effect of Hcy on platelets was prevented by antioxidants agents *in vitro* and *in vivo*. Furthermore homocysteine treatment increased platelet aggregation induced by ADP. Based on the results presented herein, we propose that inhibition of extracellular ATP and ADP hydrolysis caused by homocysteine was probably due oxidative stress, since antioxidants prevented such effects. These findings may contribute to an increase platelet response to ADP and consequence development of thrombotic risk attributed to hyperhomocysteinemia.

Intoduction

Homocysteine (Hcy) is a sulphur-containing amino acid that is closely related to the essential amino acid methionine and to cysteine [1]. Hcy is a metabolic intermediary in the transmethylation dependent of vitamin B12 and transsulfuration dependent of vitamin B6 [1,2]. In normal conditions, the plasma levels of Hcy in humans are low (5–15 μM); however, genetic defects in the enzymes that metabolize Hcy or environmental factors markedly increase Hcy in the bloodstream [3]. Mild (15-25 μM) and intermediate (25-50 μM) hyperhomocysteinemia are mainly related to acquired factors whereas the severe hyperhomocysteinemia (>100 μM) is associated to genetic defects in cystathionine beta synthase and methylenetetrahydrofolate reductase enzymes [3].

Elevated levels of plasma Hcy are recognized to be an independent risk factor for the development of atherosclerosis and thrombosis [4,5]. The possible mechanisms by which Hcy must be contributing to atherogenesis and thrombosis include increased smooth muscle cell proliferation, cytotoxicity, increased oxidative stress, stimulation of low-density lipoprotein oxidation, induction of endothelial dysfunction, enhanced coagulability and platelet activation [6,7]. In this context, recent studies have demonstrated involvement of Hcy actions linked to oxidative stress [8,9]. Besides that, some works have related Hcy effects on platelets function *in vitro* and *in vivo* [10–12]. Leoncini et al. [13] demonstrated that high plasmatic Hcy levels increased Ca^{2+} levels and reduced nitric oxide formation, a potent antiaggregating agent of platelets. In addition, studies *in vitro* have shown that Hcy enhances thromboxane A_2 (TXA_2) levels [14] and TXA_2 biosynthesis in patients with homocystinuria [15].

Platelets are fundamental elements to the thrombogenesis process. Its activation leads to release of pro-aggregating mediators, such as ADP [16]. Even at micromolar concentration, ADP is able to induce platelet aggregation *in vivo* and its hydrolysis to adenosine, an inhibitor of platelet aggregation, is required to balance the hemostatic system [17,18]. Nucleotides exert their function through binding to purinergic receptors (P2), which comprises ionotropic receptors ($P2X_{1\text{-}7}$, permeable to Na^+, K^+ and Ca^{+2}) or G-protein coupled receptors (eight subtypes, $P2Y_{1,\,2,\,4,\,6,\,11,\,12,\,13,\,14}$) [19]. Platelets express $P2Y_{12}$, $P2Y_1$ and $P2X_1$, which are involved in the shape changes, aggregation, thromboxane A_2 generation, procoagulant activity, calcium influx, adhesion and thrombus formation [20].

The importance of adenine nucleotides in homeostasis and thrombosis is greatly correlated with the essential role of an enzymatic system that provides an adequate control of these signaling molecules in the extracellular medium. Members of several families of enzymes, known as ectonucleotidases, are able to hydrolyze extracellular nucleotides to their

* Corresponding author. Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-anexo, 90035-003 Porto Alegre, RS, Brazil. Tel.: +55 51 33085554.
E-mail address: abattastini@gmail.com (A.M. Oliveira Battastini).

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respective nucleosides [21,22]. In mammals, eight related enzymes that hydrolyze extracellular tri- and diphosphonucleosides (named NTDPase1–8) have been cloned and characterized. Membrane bound NTPDase1/CD39 hydrolyzes ATP and ADP with comparable rates producing almost directly AMP a substrate for ecto-5'-nucleotidase (E.C. 3.1.3.5) with subsequent release of adenosine [21,23]. These enzymes are distributed on the membrane of different blood cells, such as leukocytes [24], endothelial cells [25] and platelets [24,26,27]. Platelet NTPDase1 is an important regulator of nucleotides-mediated signaling in a temporal and spatial manner and must contribute to thrombus formation and platelet activation [24,26–29].

Considering that Hcy is a risk factor for hypercoagulable status and the importance that adenine nucleotides play on platelets function during thrombosis process, we attempted to investigate the *in vitro* and *in vivo* effect of Hcy on the ATP, ADP and AMP hydrolysis by rat platelets.

Materials and methods

Animals and Reagents

Male Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals, 29 days old and weighing 50–100 g, were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.), at a room temperature of 22 ± 2 °C. The rats had free access to standard laboratory rat chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Platelet preparation

The platelets were prepared in accordance with the method of Lunkes and collaborators [30], with minor modifications. Total blood was collected by cardiac punction into a flask containing 120 mM sodium citrate as anticoagulant. The total blood–citrate system was centrifuged at 160 × *g* during 15 min in order to remove the residual blood cells and to obtain the platelet-rich plasma (PRP). The PRP was centrifuged at 1,400 × *g* for 20 min and washed twice by centrifugation at 1,400 × *g* for 10 min with 3.5 mM HEPES isosmolar buffer pH 7.5 containing 142 mM NaCl, 2.5 mM KCl and 5.5 mM glucose. The washed-platelets were resuspended in HEPES buffer for enzyme assays.

Enzyme assay

For the analysis of the nucleotide hydrolysis, about 20 μg of washed-platelets were preincubated for 10 min at 37 °C in a reaction mixture contained 50 mM Tris–HCl, 120 mM NaCl, 5.0 mM KCl, 6.0 mM glucose, 5.0 mM $CaCl_2$, for ATP and ADP, or 5.0 mM $MgCl_2$ for AMP, pH 7.5, in a final volume of 200 μL. The enzyme reaction was started by the addition of ATP, ADP or AMP as substrate to a final concentration of 1.0 mM. After 60 min, trichloroacetic acid (TCA) (5%, final concentration) was added to stop the reaction. The inorganic phosphate (Pi) released was measured by malachite green method [31]. Controls with the addition of the platelets after addition of TCA were used to correct nonenzymatic hydrolysis of the substrates. All samples were performed in triplicate. Protein was measured by the Coomassie blue method using bovine serum albumin as standard [32]. Specific activities were expressed as nmol Pi released per minute per milligram of protein.

In vitro experiments

The effect of different concentrations of D, L-Hcy (10, 20, 30, 50, 100 and 500 μM) was tested on ATP, ADP and AMP hydrolysis by rat platelets. The concentrations were chosen to reflect mild, intermediate and severe Hcy concentration observed in human [3]. The washed-platelets were preincubated with Hcy for 10 min and the ATP, ADP and AMP hydrolysis was measured as described above. A purified apyrase (grade VII from Sigma) activity was measure in the same assay conditions and Hcy concentrations tested in platelets. To evaluate the antioxidant effects, the washed-platelets were preincubed with 1 mM Trolox [33], a water-soluble analog of vitamin E, and Hcy (50 μM) for 10 min and the ATP, ADP and AMP hydrolysis was measured as described above.

In vivo experiments

Animals were divided in two groups as follow: group I (control), animals received saline solution (0.9% NaCl); group II (Hcy-treated), animals received a single subcutaneous dorsal injection of D,L-Hcy (0.6 μmol/g) dissolved in saline [34]. Rats were anesthetized and the blood samples were obtained by cardiac puncture 1 h after the saline or Hcy injection. The Hcy dose was calculated from pharmacokinetic parameters previously determined in our laboratory [34].

For the antioxidant treatment the animals were pretreated for 1 week with daily i.p. administration of vitamin E (alfa-tocopherol, 40 mg/kg/day) or vehicle (Tween 80) [35]. Twelve hours after the last administration, the animals were divided in four groups as follow: (I) animals pretreated with Tween 80 and received one subcutaneous dorsal injection of saline (control group); (II) animals pretreated with Tween 80 and received one subcutaneous dorsal injection of D,L Hcy (0.6 μmol/g) (Hcy group); (III) animals pretreated with vitamin E and received one subcutaneous dorsal injection of saline (Vit E group); (IV) animals pretreated with vitamin E and received one subcutaneous dorsal injection of D,L Hcy (0.6 μmol/g) (Vit E + Hcy group). All the animals were killed 1 hour later and the platelets were prepared as described above. Immediately after, the ATP, ADP and AMP hydrolysis was measured as described for the *in vitro* experiments.

Platelet aggregation

Platelet aggregation responses were monitored by turbidimetric method according to Born [36]. The blood samples were collected by cardiac puncture following 1 h the saline or Hcy injection, as described above and it were centrifuged at 160 × *g* for 15 min at room temperature to achieve a PRP suspension. Platelet aggregation experiments were performed in Aggregometer (model 490; Chrono-Log Corp., Haverton, PA) and quantified by light transmission during 3 min following the application of agonist ADP (10 μM). The aggregation was expressed as the maximal percentage change in light transmittance from baseline, using platelet-poor plasma as a reference.

Statistical analysis

All the data are expressed as the mean ± standard deviation (SD). Data were analyzed statistically by Student's *t* test or ANOVA followed by the Tukey multiple tests. Differences were considered significant when the probability was $P < 0.05$.

Results

In vitro experiments

Fig. 1 shows that physiological Hcy concentration (10 μM) did not promote alteration in the extracellular nucleotide hydrolysis when compared to the respective controls. However, Hcy in the concentration range of 20 to 500 μM caused a significant decrease on ATP (around 30%, $p < 0.05$) and ADP (around 45%, $p < 0.05$) hydrolysis, respectively (Fig. 1A and B), while AMP hydrolysis was not altered (Fig. 1C) in none Hcy concentration tested. To test whether the inhibitory effect of Hcy on ATP/ADP hydrolysis was dependent on

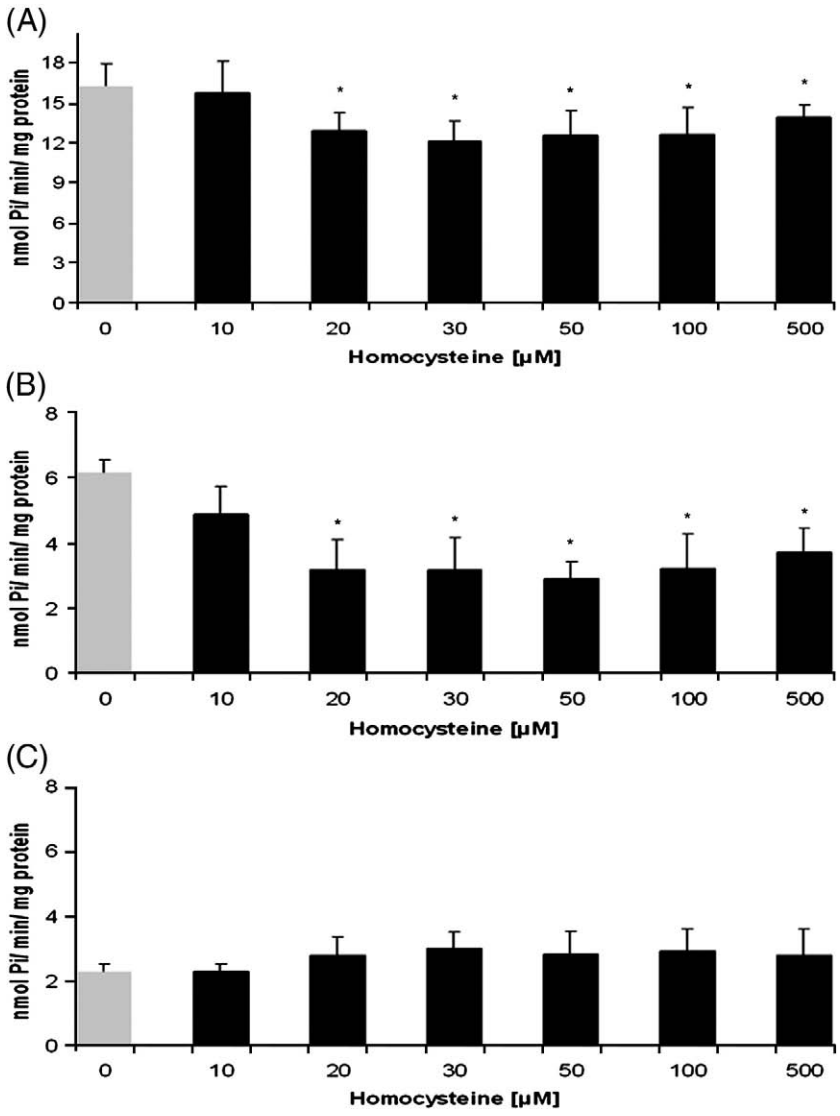

Fig. 1. *In vitro* effect of different concentrations of Hcy on nucleotide hydrolysis by rat platelets. The platelets were incubated with increasing concentrations of Hcy (10, 20, 30, 50, 100 and 500 µM) and the enzyme reaction was started by the addition of ATP (A), ADP (B) or AMP (C) to final concentration of 1 mM as described in Material and Methods. The control activities were 16.18 ± 1.64, 6.16 ± 0.4 and 2.28 ± 0.26 nmol Pi/min/mg protein for ATP, ADP and AMP, respectively. The data represent mean ± S.D. (n = 8). Statistical analysis was performed by ANOVA, followed by Tukey's test. * Represent significant statistical difference compared to the respective control group, considering $p < 0.05$ as significant. Hcy- Homocysteine.

platelet cell system, we examined the effect of Hcy on a purified apyrase activity, which hydrolyzes ATP and ADP at the same rate as NTPDase1 [37]. Hcy did not inhibit the hydrolysis of ATP and ADP by apyrase at the same concentrations tested in platelets (data not shown), suggesting an indirect effect of Hcy. Considering that a possible pro-oxidative effect could be involved in the nucleotide hydrolysis inhibition caused by Hcy, we investigate the effect of Trolox on the inhibitory effect of Hcy on ATP and ADP hydrolysis. Fig. 2 shows that the inhibition on ATPase/ADPase activities by Hcy was significantly reverted by Trolox ($p < 0.05$).

In vivo experiments

In order to determine the *in vivo* effect of Hcy on nucleotide hydrolysis by rat platelets, the animals received a single subcutaneous dorsal injection of D,L-Hcy (0.6 µmol/g) and the platelets were isolated and submitted to enzyme assay, as described in materials and methods. The results showed that the acute Hcy administration on nucleotide hydrolysis exhibit a similar pattern of inhibition to the *in vitro* results. Hcy administration promoted a decrease in ATP (39%, $p < 0.05$) and ADP (38%, $p < 0.05$) hydrolysis when compared to controls, while the AMP hydrolysis was unaltered (Fig. 3). Rats pretreated with vitamin E prevented this Hcy inhibitory effect of the ATP/ADP hydrolysis ($p < 0.05$), as shown in Fig. 4.

Platelet aggregation

To verify whether the Hcy administration could influence platelet function, we used the PRP in the aggregation study. The Fig. 5 shows that a single injection of Hcy increased platelet aggregation induced by ADP when compared to controls ($p < 0.05$). In addition, Hcy did not induce the spontaneous platelet aggregation when it was added to a PRP (data not shown).

Discussion

Hyperhomocysteinemia is an independent risk factor for athero-thrombotic disease. The mechanism by which homocysteine induces atherosclerosis and thrombosis is not fully understood. A number of *in*

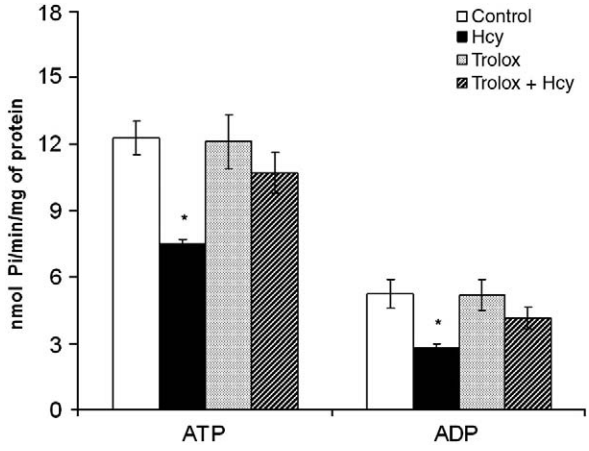

Fig. 2. *In vitro* effect of Trolox on ATP and ADP hydrolysis in rat platelets incubated with Hcy. The platelets were incubated simultaneously with 1 mM Trolox and 50 μM Hcy and the enzyme reaction was started by the addition of ATP or ADP to final concentration of 1 mM as described in Material and Methods. The control activities were 12.3 ± 0.74, and 5.26 ± 0.64 nmol Pi/min/mg protein for ATP and ADP, respectively. The data represent mean ± SD (n = 6). Statistical analysis was performed by ANOVA, followed by Tukey's test. * Represent significant statistical difference compared to the respective control group, considering $p < 0.05$ as significant. Hcy- Homocysteine.

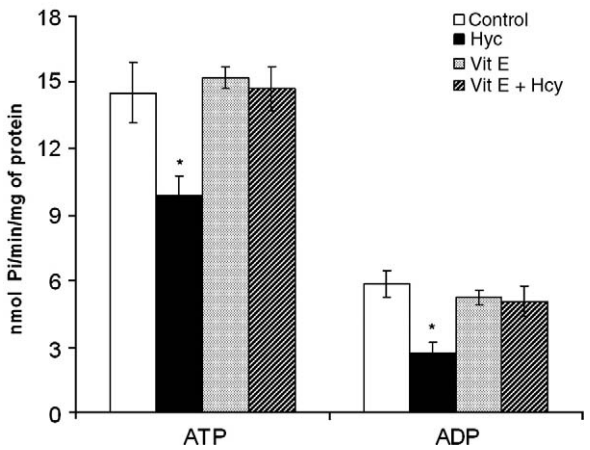

Fig. 4. Effect of Hcy administration on ATP and ADP hydrolysis in platelets of rats pretreated for 1 week with vitamin E. The platelets from rats of indicated groups were incubated with ATP, ADP or AMP to final concentration of 1 mM as described in Material and Methods. The control of enzymatic activities in platelets was 14.51 ± 1.37 and 5.86 ± 0.61 nmol Pi/min/mg protein for ATP and ADP respectively. The data represent mean ± S.D. (n = 6). Statistical analysis were performed by ANOVA, followed by Tukey's test. * Represent significant statistical difference compared to the respective control group, considering $p < 0.05$ as significant. Hcy – Homocysteine; Vit E - vitamin E.

vitro and *in vivo* effects of Hcy on vascular endothelium, platelets and coagulation have been described, which may be related to vascular disease [2,3,5,12,15].

The present study investigated whether Hcy *in vitro* or *in vivo* (acute Hcy administration) causes alteration in the extracellular hydrolysis of adenine nucleotides on platelets. *In vitro* results demonstrated that Hcy inhibited ATP and ADP hydrolysis in the concentrations of 20 μM to 500 μM, but not at 10 μM (physiological concentration) (Fig. 1). Alexandru et al. [38] using preparations of washed platelets showed that Hcy was able to induce endogenous generation of reactive oxygen species (ROS) on platelets of healthy individuals. Our data *in vitro* demonstrated that addition of Trolox, a free radical scavenger, prevented the inhibitory effect of Hcy on ATPase/ADPase activities (Fig. 2). These results indicate that enhanced oxidative stress is probably related to the observed inhibitory effect of Hcy. So, it is likely that an increased formation of reactive oxygen species results in increased oxidative damage in membrane lipids and structures linked to it. Previous studies have demonstrated that Hcy increases lipid peroxidation *in vitro* [9] and reduces radical-trapping antioxidant potential. In addition, Matté et al. [8] showed that Hcy administration reduced antioxidant potential in cerebral and plasma of rats.

It was previously showed that acute Hcy administration decreased TRAP, Na^+, K^+-ATPase and catalase (CAT) activities by 20%, 60% and 15%, respectively in hippocampus of rats [35]. They also showed that vitamins E and C completely prevented the action of Hcy on TRAP, Na^+,K^+-ATPase and CAT activities, indicating the participation of oxidative stress is probably involved in the Hcy effects. In addition, it was recently showed that chronic homocysteine administration increased DNA damage and disrupted antioxidant defenses (enzymatic and non-enzymatic) in parietal cortex and blood plasma [8]. In this last study it was used an experimental rat model of hyperhomocysteinemia [34], where plasma Hcy concentration achieves levels similar to those found in human. It is important to observe that in these two studies the levels of enzymatic inhibition as well as the effects on the antioxidant defenses were at 20 - 40% of inhibition, comparable to the effects of Hcy on ATP/ADP

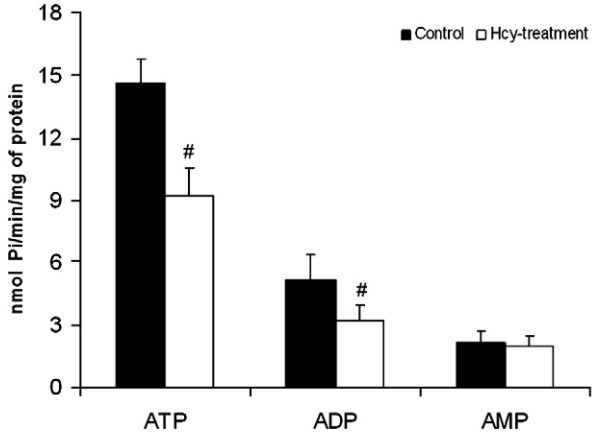

Fig. 3. Nucleotide hydrolysis in platelets from rats after 1 h of Hcy administration. The platelets from rats of indicated groups were incubated with ATP, ADP or AMP to final concentration of 1 mM as described in Material and Methods. The control of enzymatic activities in platelets was 14.65 ± 1.15, 5.2 ± 1.1 and 2.17 ± 0.53 nmol Pi/min/mg protein for ATP, ADP and AMP, respectively. The data represent mean ± S.D. (n = 8). Data were analyzed by Student's *t* test for independent samples. # Represent significant statistical difference compared to the respective control group, considering $p < 0.05$ as significant.

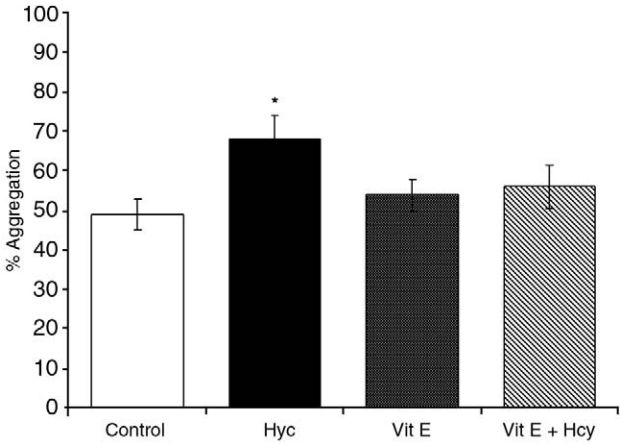

Fig. 5. Effect of Hcy administration on the platelet aggregation induced by ADP (10 μM). The PRP was obtained from rats 1 h after Hcy injection. Platelet aggregation was expressed as the maximal percent change in light transmittance within 3 min from baseline. The data represent mean ± S.D. (n = 10). Statistical analysis were performed by ANOVA, followed by Tukey's test, considering $p < 0.05$ as significant (*). Hcy - Homocysteine.

hydrolysis showed here, even when we tested 500 μM Hcy in the *in vitro* experiments. So, it is possible to suggest that the Hcy induces oxidative stress that is concentration limited and that the inhibition by Hcy observed in our results could be due to the limited redox targets in the platelets and especially in the enzyme(s) responsible for ATP and ADP hydrolysis.

Data from the literature indicate that the NTPDase1 (ecto-apyrase) is the enzyme responsible for the hydrolysis of ATP and ADP in the membrane surface of platelets [24,26–29]. However the presence of other NTPDases cannot be completely excluded considering that the *in vitro* effect of Hcy was quite different on ATPase and ADPase activities. In this sense, considering that platelet NTPDases are integral membrane proteins, with two transmembrane domains, which are important to the enzymatic activity [21,39] it is plausible to suggest an indirect effect of Hcy through disequilibrium in the redox state of the platelets, which may alter the normal function of this enzyme in the platelets exposed to Hcy. This hypothesis was reinforced by the results with purified apyrase, since Hcy did not affect the ATP and ADP hydrolysis catalyzed by this soluble enzyme, indicating the involvement of platelet membrane in the inhibition of nucleotide hydrolysis caused by Hcy.

It is well documented that Hcy auto-oxidizes in plasma and generate reactive oxygen species [40–42] and that Hcy is able to induce oxidative stress on platelets [43]. Frasseto et al. [44] demonstrated that oxygen radicals are involved in decreased ATP/ADP hydrolysis on rat platelets. In addition, Krotz et al. [45] suggested an oxidative damage to platelet CD39 (NTPDase1) to justify the increased sensitivity to the superoxide anion in the aggregation induced by ADP when compared to control conditions. Inactivation of NTPDase1 by superoxide anion, resulting in an increased ADP concentration, was also described by Robson et al. [46] in endothelial cells. In the *in vivo* experiments, we showed that the pretreatment of animals with Vitamin E, a lipid-soluble antioxidant that acts as a defense against oxidative stress [47], prevented the inhibition of ATP/ADP hydrolysis caused by the Hcy administration (Fig. 4). These data corroborate the hypothesis that unbalanced oxidative status is related to the inhibitory effect of Hcy on ATPase/ADPase activities.

Consistent with previous reports, we did not find any alteration in the platelet aggregation when only Hcy was added to the PRP (data not shown) demonstrating that there was not spontaneous platelet aggregation only with Hcy presence [7,10]. On the other hand, studies have demonstrated increased platelet sensitivity Hcy-induced to agonists in animals models [48,49], as observed in our results (Fig. 5). So, we suggest that the inhibition of ADP hydrolysis after Hcy exposition could result in an increase in the reactivity and aggregability of platelets, with an ADP accumulation in the extracellullar milleu when this agonist is added to platelets (Fig. 5). Otherwise, ATP, a competitive inhibitor of ADP [50], can also accumulate after NTPDase1 inhibition, and, at least in part, could be limiting the spontaneous platelet aggregation. Furthermore, during vessel damage high levels of pro-aggregates substances such as ADP, are released to the plasma [51], which could trigger thrombus formation in pathological states. Therefore, in this scenario the NTPDase1 platelet inhibition, in consequence of Hcy exposition, could result in increased platelet response to ADP and in this way to represent an additional risk factor to thrombogenesis associated to high level of Hcy.

Finally, the Hcy effects have a multi-factorial nature and the increased platelet reactivity can be a consequence of many confluent mechanisms such as reduced NO formation, increased Ca^{++}, increased thromboxane A_2 [13–15] and decrease in the scavenger of ADP.

Based on the results presented herein, we propose that inhibition of platelet NTPDase1 caused by homocysteine was probably due oxidative stress, since antioxidants prevented such effects. Taken together, these findings may contribute to development of thrombotic risk attributed to hyperhomocysteinemia.

Conflict of interest statement

No conflict of interest exists in the present study.

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4. DISCUSSÃO

A sinalização purinérgica compreende nucleotídeos e seus respectivos nucleosídeos, receptores específicos do tipo P1 e P2 e as ectonucleotidases. Dentre as ectonclueotidases encontramos as enzimas que hidrolisam os nucleotídeos tri- e difosfato (ENTPDases/CD39) e a ecto-5´-nucleotidase/CD73 que hidrolisa os nucleosídeos monofosfato (em especial o AMP) as quais exercem profunda atividade modulatória mantendo o equilíbrio e disponibilidade desses ligantes no meio extracelular (Zimmermann, 2001; Robson *et al.,* 2006). O sistema purinérgico, assim organizado, possui ações em diversos sistemas biológicos com especial atenção ao sistema vascular e mais recentemente ao sistema imuno-inflamatório (Ia Sala *et al.*, 2003; Marcus *et al.,* 2003: Bours *et al.,* 2006; Stagg *et al.,* 2010). No sistema vascular, a sinalização purinérgica envolve principalmente as células endoteliais e a ativação de plaquetas estando dessa forma diretamente relacionada aos processos trombogênicos (Marcus *et al.,* 2003). Já no campo inflamatório, o sistema purinérgico envolve a migração de leucócitos, a secreção de citocinas em macrófagos além de processos fagocitários (Bours *et al.,* 2006; Dwyer *et al.,* 2007; Hyman *et al.,* 2009). Portanto, elementos que interfiram na arquitetura dessa sinalização podem resultar em prejuízos, os quais podem desencadear ou contribuir para o desenvolvimento de processos patológicos.

Com o prévio conhecimento a respeito da plasticidade dos macrófagos durante o desenvolvimento da resposta inflamatória, nosso primeiro passo foi traçar o perfil das ectonucleodidases bem como a presença dos receptores purinérgicos nos diferentes fenótipos de ativação de macrófagos, com objetivo de determinar ou avaliar o

comportamento do sistema purinérgico nos macrófagos induzidos à diferenciação (Item 3.1). Para tanto, os macrófagos foram diferenciados em dois fenótipos: um fenótipo pró-inflamatório (também conhecido como macrófagos classicamente ativados ou M1) através da estimulação por LPS e um fenótipo antiinflamatório (também conhecido como macrófagos alternativamente ativados ou M2) através da estimulação por IL-4.

Os Macrófagos obtidos do peritônio de camundongos foram cultivados e estimulados com LPS ou IL-4. Após o estímulo as populações de macrófagos foram caracterizadas através da análise de parâmetros bioquímicos pela atividade da iNOS e arginase, perfil de expressão de RNAs mensageiros (FIZZ1 e Ym1) e pela secreção de citocinas. Os macrófagos residentes apresentaram uma atividade baixa das enzimas marcadoras de diferenciação, iNOS e arginase enquanto que os macrófagos tratados com LPS exibiram um aumento da atividade de iNOS e uma atividade reduzida de arginase. Em contraste, macrófagos tratados com IL-4 mostraram um atividade de iNOS diminuída e uma atividade aumentada de arginase (Item 3.1, Figura 1A). Dados da literatura demonstram ainda que altos níveis de expressão dos genes *Retnla* (FIZZ1) e *Chi3l3* (Ym1) constituem marcadores úteis para identificação de macrófagos alternativamente ativados (M2) tanto *in vitro* como *in vivo* (Raes *et al.*, 2002). Nossos resultados mostram uma alta expressão desses marcadores somente para macrófagos tratados com IL-4, confirmando o fenótipo alternativo (M2) (Item 3.1, Figura 1B). Por fim, como esperado, os macrófagos tratados com LPS secretaram níveis aumentados da citocina pró-inflamatória TNF-α em relação aos tratados com IL-4. Por outro lado, a produção da citocina anti-inflamatória IL-10 foi maior nos macrófagos tratados com IL-4 em relação aos tratados com LPS (Item 3.1, Figura 1C). Portanto, levando em conta os resultados descritos acima podemos assegurar que os macrófagos foram

diferenciados em dois fenótipos extremos: um fenótipo pró-inflamatório (M1) e um fenótipo anti-inflamatório (M2), os quais foram utilizados para os estudos subsequentes desta tese.

A caracterização das ectonucleotidases demonstrou a presença da E-NTPDase1 nos macrófagos residentes, e estimulados com LPS e IL-4 com diferente padrão de expressão. Além disso, nós também demonstramos a presença de E-NTPDase3 e ecto-5'-nucleotidase nos três fenótipos (Item 3.1, Tabela 2).

Os macrófagos ativados com LPS apresentaram uma redução significativa na atividade ATPásica (~20%) em relação aos macrófagos residentes. Entretanto, de maneira surpreendente, não houve alteração na hidrólise de ADP de macrófagos estimulados com LPS em relação às células residentes, o que merece uma melhor avaliação, pois a princípio, a hidrólise do ADP e do ATP por uma ENTPDase1 deveria sofrer efeitos paralelos como demonstrado em muitos outros estudos (Leal *et al.*, 2005; Rico *et al.*, 2008; Santos *et al.*, 2009). Já em relação aos macrófagos ativadas com IL-4, os macrófagos estimulados com LPS apresentaram uma diminuição significativa de aproximadamente 40% na hidrólise de ATP e 30% na hidrólise de ADP. A análise em HPLC confirmou o resultado e demonstrou uma menor degradação de ATP pelos macrófagos estimulados com LPS em relação às células residentes e estimuladas com IL-4. Além disso, a análise dos produtos de degradação do ATP por HPLC demonstra claramente um perfil compatível com a presença de uma ENTPDase1 e/ou ENTPDase3, uma vez que não é observado acúmulo de ADP ao longo da incubação, sendo produzido quase que diretamente o AMP o qual é subseqüentemente hidrolisado à adenosina (Artigo 1, Figura 4). Os dados indicaram também uma diminuição significativa na expressão do RNA mensageiro e das ENTPDase1 e ENTPDase3 na membrana de

macrófagos ativados por LPS (Item 3.1, Figura 5 e 6). Estudos prévios relatam a perda na atividade da E-NTPDase1 em células endoteliais após serem expostas ao LPS (Kittel *et al.,* 2007). A redução na atividade ATPásica observada no presente trabalho pode ser explicada por alterações na membrana devido aos mecanismos relacionados à inflamação ocasionada por LPS, visto que a atividade da E-NTPDase1 é susceptível a mudanças no seus domínios transmembrana e também à mudança nas propriedades da membrana na qual ela está ancorada (Grinthal *et al.,* 2007; Kitte *et al.,* 1999; Wang *et al.,* 1998). Esta explicação pode ser expandida para as outras E-NTPDases, já que essas enzimas são sensíveis a mudanças nos domínios transmembrana também. Portanto, nossos resultados podem ser, em parte, justificados pela perturbação causada pelo LPS na membrana, porém, modificações transcricionais e/ou pos-trancricionais devem estar envolvidas também na ativação por LPS. Além disso, essa redução transitória da hidrólise de ATP no fenótipo pró-inflamatório (M1) de macrófagos pode ser benéfica, visto que o ATP pode estimular a depuração (destruição) de patógenos. Para contribuir com nossos achados, o receptor purinérgico pró-inflamatório $P2X_7$ tem sua expressão aumentada durante a ativação clássica por IFNγ, IL-1, TNFα e LPS (Humphreys *et al*., 1996;1998; Lemaire *et al.,* 2003). A ativação do receptor $P2X_7$ pelo ATP citotóxico (concentrações acima de 100 uM) poderia, dessa forma, contribuir para os efeitos pró-inflamatórios dos macrófagos. Por fim, macrófagos estimulados com IL-4 mostraram atividades ATPásicas e ADPásicas significativamente aumentadas em relação ao macrófagos residentes e estimulados com LPS (Item 3.1, Figuras 2 e 3). Soma-se a isso, o aumento da expressão das E-NTPDase1 e E-NTPDase3 tanto em nível de RNA mensageriro quanto de proteína (Item 3.1, Figuras 5 e 6). Portanto, os macrófagos estimulados com IL-4 tem uma maior atividade ATP/ADPásica que pode conduzir

mais rapidamente o ATP até adenosina, a qual tem importante atividade antiinflamatória, como discutido a seguir.

A adenosina extracelular, um metabolito produzido principalmente pela degradação sequencial do ATP, pode estar elevada durante o processo infamatório e é associada a ações antiinflamatórias e de regeneração em células imunes (Haskó *et al.*, 2004;2009). Nossos resultados demonstraram que o LPS diminui a atividade da ecto-5'-nucleotidase, principal enzima envolvida na etapa final de produção de adenosina, quando comparado a macrófagos residentes e estimulados com IL-4 sendo essa menor atividade enzimática acompanhada pela redução no nível de RNA mensageiro e proteína na superfície das células (Item 3.1, Figuras 2, 5 e 6). Estudos têm revelado que a inibição de um importante fator de transcrição, o NF-κB aumenta a atividade basal da ecto-5'-nucleotidase em uma linhagem de células endoteliais de cordão umbilical humano (HUVEC) (Li *et al.*, 2008). A estimulação de macrófagos com LPS acarreta na ativação de varias fatores de transcrição como, por exemplo, o NF-κB. Portanto, é plausível sugerir que o NF-κB poderia diminuir a transcrição do gene que codifica a ecto-5'-nucleotidase. Está hipótese é respaldada por estudos que reportam que o efeito antiinflamatório do Metotrexato é resultado da ativação da ecto-5'-nucleotidase via supressão de NF-κB (Montesinos *et al.*, 2007). Além disso, a redução na hidrólise de AMP foi acompanhada pela diminuição da proteína ecto-5'-nucleotidase na superfície das células, indicando que a atividade enzimática é proporcional a quantidade de enzima presente na membrana das células. Portanto, nossos resultados demonstram que a perda na atividade da ecto-5'-nucleotidase ocorreu em nível molecular e protéico. Outro resultado que chama a atenção foi a presença de níveis sustentáveis de adenosina após a hidrólise de AMP, como mostra a análise por HPLC, em macrófagos estimulados por IL-4 (Item 3.1, Figura 4). Isso pode ocorrer devido, principalmente, a dois fatores: o

primeiro, uma maior expressão e atividade da ecto-5'-nucleotidase (AMP → ADO); segundo, poderia ser resultado da diminuição na degradação da adenosina até inosina ou ainda devido a ambos. A segunda hipótese não deve ser a mais provável uma vez que os níveis de inosina são mantidos inalterados nas três populações de macrófagos estudadas. Além disso, embora, a participação da inosina tenha sido sugerida em processos inflamatórios, pouca são as evidências detalhando seu mecanismo de ação em nível celular (Haskó *et al.,* 2000;2004). Por outro lado, a adenosina tem suas ações mais bem definidas em seus receptores como uma potente molécula sinalizadora que participa na regulação de muitos processos fisio-patológicos como a ativação de macrófagos (Haskó *et al.,* 2004; Bours *et al.,* 2006). Nesse contexto, nos podemos sugerir que os níveis sustentáveis de adenosina em macrófagos estimulados com IL-4 seriam mais relevantes para as ações antiinflamatórias e de regeneração do fenótipo M2 dos macrófagos.

Finalmente, nós mostramos nesse primeiro trabalho (Item 3.1) que os receptores purinérgicos P1 e P2 apresentaram o mesmo perfil de expressão de RNA mensageiro nos 3 fenótipos estudados. Assim, de uma forma geral, podemos dizer, muito provavelmente, que a mudança no perfil de expressão das enzimas E-NTPDase1, E-NTPDase3 e ecto-5'-nucleotidase em macrófagos durante a diferenciação fenotípica deva dirigir a sinalização para uma cascata purinérgica que conduz para uma progressiva diminuição na concentração de nucleotídeos e um aumento na disponibilidade de nucleosídeos (adenosina). Portanto, tais mudanças na funcionalidade das ecto-enzimas e não nos receptores, devem permitir aos macrófagos o ajuste da cascata purinérgica na sua superfície, de maneira a manter um rígido controle das funções efetoras durante os eventos inflamatórios.

Tendo em vista que níveis séricos aumentados de homocisteína, um intermediário formado durante o metabolismo da metionina, é um importante fator de risco associado ao desenvolvimento de aterosclerose e trombose, o próximo passo desta tese foi avaliar os efeitos da homocisteína sobre o sistema purinérgico de macrófagos e plaquetas. Assim, após termos verificado o perfil das E-NTPDases e da ecto-5'-nucleotidase nos diferentes fenótipos de ativação dos macrófagos, nós avaliamos se concentrações micromolares de homocisteína (50μM e 100μM), as quais podem ser encontradas em indivíduos com elevados níveis de homocisteína (hiperhomocisteinemia), poderiam ter efeito sobre a diferenciação de macrófagos e sobre as enzimas envolvidas na hidrólise dos nucleotídeos (Item 3.2).

Uma importante característica no desenvolvimento da aterosclerose é o acumulo de macrófagos na lesão. Nesse trabalho (Item 3.2) nós mostramos que macrófagos tratados com homocisteina polarizam os mesmos para um fenótipo pró-inflamatório (M1). Esta polarização foi definida pela produção de citocinas, atividade da iNOS e da arginase e pelo nível de RNA mensageiro de FIZZ1 e Ym1 (Item 3.2, Figura 1). De acordo com dados prévios (Woo *et al.*, 2003; Holven *et al.*, 2006; Martinez *et al.*, 2008), nossos resultados demonstram que a homocisteína tem potente propriedade pró-inflamatória sobre os macrófagos. No nosso sistema de estudo, a homocisteína aumentou a citocina TNF-α e não aumentou a produção de IL-10. O fenótipo pró-inflamatório foi demonstrado também pelo fato que a homocisteína aumentou a produção de nitrito, medida de indireta de atividade da iNOS, enquanto não afetou a atividade da arginase. Por fim, a homocisteína não alterou a expressão de RNA mensageiro do Ym1 e FIZZ1, os quais são fortes marcadores de ativação do tipo M2 (ativação alternativa). Este perfil polarizado pro-inflamatório dos macrófagos deve

contribuir para desenvolvimento da aterosclerose que está relacionada às concentrações aumentadas de homocisteína.

O ATP e seus produtos de degradação como a adenosina representam um importante papel em processos inflamatórios envolvendo macrófagos (Bours *et al.*, 2006). Nossos resultados demonstraram também que a exposição de macrófagos a homocisteína aumentou significativamente a hidrólise de ATP e ADP quando comparado as células residentes (Item 3.2, Figura 2). Os resultados ainda revelam que não houve diferença entre as concentrações de homocisteína analisadas (50µM and 100µM). As análises de HPLC confirmaram os resultados da atividade de hidrólise dos nucleotídeos, medidas através da quantificação do fosfato inorgânico liberado, e mostram que a degradação do ATP é mais rápida em macrófagos tratados com homocisteína do que nos residentes (Item 3.2, Figura 3). Por outro lado, a análise quantitativa da expressão do RNA mensageiro por PCR em tempo real falhou em demonstrar diferença na expressão das E-NTPDases1 e E-NTPDase3 nos macrófagos expostos à homocisteína em relação aosresidentes (Item 3.2, Figura 5). Além disso, a homocisteína não alterou o nível de expressão da proteína E-NTPDase1, porém causou um aumento na expressão protéica da E-NTPDase3 quando comparado aos macrófagos residentes (Item 3.2, Figura 6). Esses dados somados ao aumento observado na razão das atividades ATPásica:ADPásica quando os macrófagos foram expostas a homocisteína nos leva a sugerir que a principal ecto-enzima alterada nessas células pelo tratamento com homocisteína é a E-NTPDase3.

Outro importante resultado encontrado nesse segundo artigo (Item 3.2) é que o tratamento com homocisteína aumentou a hidrólise de AMP quando comparado às células residentes (Item 3.2, Figura 2). De acordo com os primeiros dados, a análise por

HPLC mostrou que o AMP foi totalmente metabolizado em 60 minutos nos macrófagos tratados com homocisteína enquanto que nos residentes isso aconteceu somente ao final de 120 minutos de incubação (Item 3.2, Figura 3). O aumento observado na hidrólise de AMP não foi relacionado à expressão do RNA mensageiro e nem à expressão da ecto-5'-nucleotidase na membrana celular (Item 3.2, Figura 5 e 6). Chama a atenção também o fato de que os níveis de inosina, produto final da degradação extracelular do ATP, estão diminuídos nos macrófagos tratados com homocisteína (Item 3.2, Figura 3).

Em conjunto, os resultados desse segundo trabalho demonstraram mudanças na atividade ATPásica, ADPásica e AMPásica em macrófagos tratados com homocisteína e que apresentam características pró-inflamatórias. Considerando que altos níveis de nucleotídeos extracelulares estão presentes em danos celulares (lise cellular), degranulação de plaquetas e locais de inflamação, as ectonucleotidases, tais como as E-NTPDases e a ecto-5'-nucleotidase, desempenham um papel crucial no controle dos níveis de nucleotídeos e nucleosídeos no microambiente das células imunes durante a inflamação (Atkison *et al.,* 2006; Bours *et al.,* 2006; Robson *et al.,* 2006). Então, a rápida degradação de ATP e ADP, propiciada pelo tratamento com homocisteína simultaneamente com o aumento da atividade da ecto-5'-nucleotidase poderia promover uma cascata enzimática completa que permite a hidrólise de ATP até adenosina, que é classicamente conhecida como uma molécula com ações antiinflamatórias em células imunes. Considerando que os macrófagos tratados com homocisteína apresentam característica de um fenótipo pró-inflamatório (M1), uma possível explicação para este resultado aparentemente contraditório seria que a adenosina gerada pela rápida hidrólise de ATP, ADP e AMP em macrófagos tratados com homocisteína é captada para o interior desses macrófagos onde, devido ao aumento das concentrações intracelulares de

homocisteína, se liga à mesma formando adenosilhomocisteína (AdoHcy) pela inversão do sentido da reação da S-adenosilhomoisteina (SAH) hidrolase conforme ilustrado na (Figura 7).

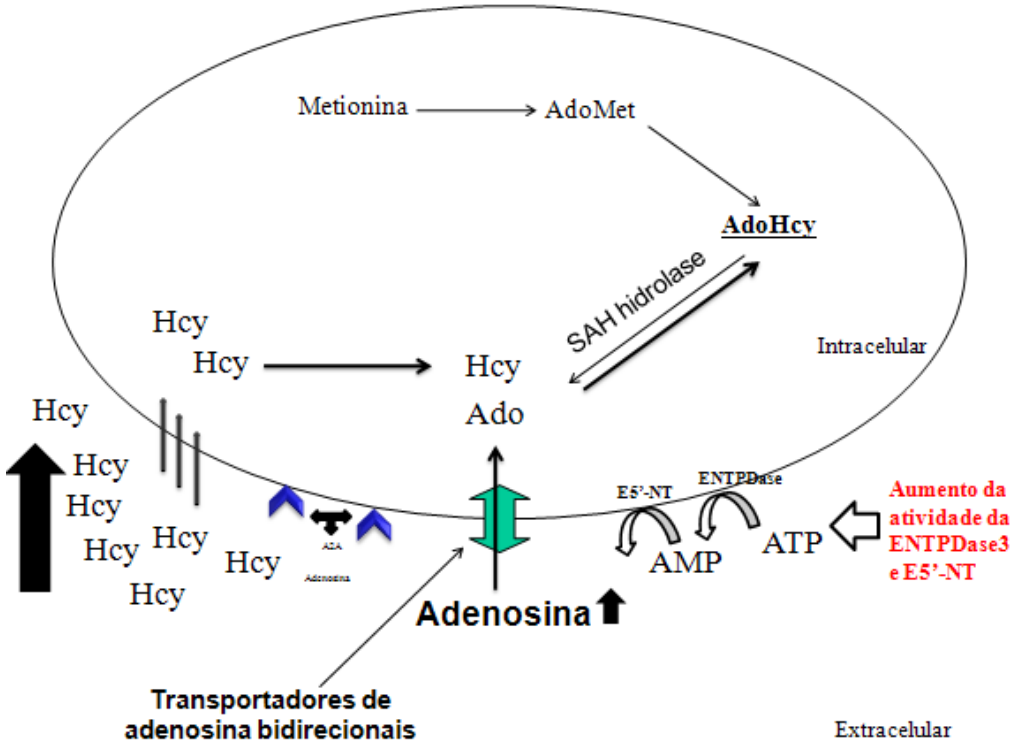

Figura 7. Representação simplificada para nossa hipótese. Durante o processo de metilação, *S*-adenosilmetionina (AdoMet) derivado da metionina é convertido em *S*-adenosilhomocisteína (AdoHcy), o qual é hidrolisado para produzir homocisteína (Hcy) e adenosina via SAH hidrolase. A reação envolvendo a hidrolise de S-adenosilhomocisteína para formar homocisteína e adenosina é reversível. Embora a constante de equilíbrio desta reação favoreça a síntese de AdoHcy, sobe condições fisiológicas AdoHcy é hidrolisada a homocisteína e adenosina. Na hiperhomocisteinemia, a reação muda para a síntese AdoHcy com consumo de adenosina intracelular. Ado- Adenosina; E5'-NT – ecto-5'-nucleotidase; A2A – receptoror de adenosina do tipo A2A (Ueland *et al.,* 1982; Undas *et al.,* 2005)

De fato, estudos prévios demonstram que altas concentrações de homocisteína na corrente circulatória ou em tecidos induz a uma queda da adenosina extracelular via transportador de nucleosídeos (Chen *et al.,* 2002; Riksen *et al.,* 2005). Portanto, nossos resultados indicam que a diminuição no nível de geração de inosina em macrófagos tratados com homocisteína (Item 3.2, Figura 3) pode estar relacionado ao aumento da

captação de adenosina do meio extracelular, levando a uma menor capacidade da adenosina em exercer seus efeitos antiinflamatórios sobre os receptores A_{2A} dos próprios macrófagos (Haskó *et al.,* 2004;2009). Dessa forma, essa condição poderia auxiliar na manutenção de um estado pró-inflamatório nos macrófagos e contribuir para as complicações inflamatórias na placa aterosclerótica associadas à hiperhomocisteinemia. Além disso, os níveis aumentados de TNF-α e da produção de nitrito aqui demonstrados pela ação da própria homocisteína comprovam o caráter pró-inflamatório dos macrófagos nessas células (Item 3.2, Figura 1).

Considerando que potencias mecanismos têm sido implicados nos processos patológicos envolvendo altos níveis de homocisteína que incluem a formação de espécies reativas de oxigênio e a ligação da homocisteina ou seus metabólitos a proteínas (homocisteinilação) (Alvarez-Maqueda *et al.*, 2004), nós testamos o efeito do trolox, um *"scavenger"* de radicais livres, na exposição da homocisteína sobre as ecto-nucleotidases. Os resultados mostraram que o trolox não causou nenhum efeito sobre a hidrólise do ATP e do ADP pela homocisteína, descartando um possível envolvimento do estresse oxidativo nesses efeitos. Por outro lado, a adição de trolox, preveniu o aumento da atividade da ecto-5'-nucleotidase em macrófagos expostos a homocisteína. Este resultado deve indicar que a formação de radicais livres está envolvida nos efeitos da homocisteína relacionados ao aumento na atividade da ecto-5'-nucleotidase.

Portanto, os resultados apresentados nesse trabalho (Item 3.2) demonstram que os macrófagos expostos a homocisteína apresentam um perfil pró-inflamatório e um aumento na hidrólise do ATP, ADP e AMP, com evidências do envolvimento da E-NTPDase3 e da ecto-5'-nucleotidase nas mudanças inflamatórias associadas a hiperhomocisteinemia em macrófagos.

Em conclusão, nossos resultados demonstram que embora os macrófagos estejam diferenciados (polarizados) para um mesmo fenótipo tanto pela ativação com LPS quanto por homocisteína, o perfil de hidrólise e de expressão das E-NTPDases e da ecto-5'-nucleotidase não convergem para a mesma direção. Portanto o controle dessas enzimas, que são fundamentais para as repostas celulares dependentes de nucleotídeos e nucleosídeos, demonstram ser bem mais complexos do que podemos ter avaliado no presente estudo. Assim, embora possamos ter o mesmo fenótipo, no caso o pró-inflamatório, a origem e o tipo de estímulo podem e devem estar relacionados às mudanças de atividade e expressão das ectonucleotidases avaliadas, com o objetivo de tentar combater/amenizar os danos resultantes dos insultos propiciados por estes estímulos.

Por fim, considerando que a homocisteína é fator de risco para associado a hipercoagulação e conhecendo a importância que os nucleotídeos representam sobre a função plaquetária, nós avaliamos a exposição de níveis elevados de homocisteína em sobre a hidrólise de ATP, ADP e AMP em plaquetas (Item 3.3).

Como mencionado anteriormente, a hiperhomocisteinemia é um fator independente de risco para doenças atero-trombóticas (Wierzbicki *et al.,* 2007). O mecanismo pelo qual a homocisteína induz aterosclerose e trombose não é inteiramente conhecido. Vários efeitos da homocisteína têm sido descritos sobre o endotélio vascular, plaquetas e coagulação sanguínea, tanto *in vitro* como *in vivo*, os quais devem ser relacionados a doenças vasculares (Leoncini *et al.*, 2003; Riba *et al.,* 2004; Mohamed *et al.,* 2007; Perla-Kaján *et al.,* 2007). No terceiro artigo (Item 3.3) dessa tese investigamos os efeitos do tratamento *in vitro* e *in vivo* (administração aguda de homocisteína) sobre a hidrólise extracelular de nucleotídeos em plaquetas.

Os resultados *in vitro* demonstraram que a homocisteína inibiu a hidrólise de ATP e ADP nas concentrações de 20 μM até 500 μM, mas não alterou em 10 μM (concentração fisiológica) (Item 3.3, Figura 1). Alexandru e colaboradores (2008) usando preparado de plaquetas mostraram que a homocisteína foi capaz de induzir a geração endógena de espécies reativas de oxigênio (EROs) sobre plaquetas de indivíduos saudáveis. Nossos dados *in vitro* demonstraram que a adição de trolox, preveniu o efeito inibitório da homocisteína sobre as atividades ATP/ADPásicas (Item 3.3, Figura 2). Esses resultados indicam que o aumento do estresse oxidativo está provavelmente relacionado aos efeitos inibitórios observados da homocisteína. Então, é provável que um aumento na formação de EROs resulte em um aumento no dano oxiadativo nos lipídeos de membrana e as estruturas ligadas a ela. Estudos recentes têm revelado que a homocisteína aumenta a peroxidação lipídica (Streck *et al.*, 2003) *in vitro* e reduz o potencial o antioxidante de plaquetas e, além disso, trabalhos tem mostrado que a administração de homocisteína reduz o potencial antioxidante em cérebro e plasma de ratos (Matté *et al.*, 2009).

Dados anteriores mostram que a administração aguda de homocisteína diminui o TRAP, a atividade da Na+, K+-ATPase e da catalase (CAT) em 20%, 60% e 15%, respectivamente, em hipocampo de ratos (Wyse *et al.*, 2003). Esses dados também revelaram que a vitamina E e C preveniram completamente a ação da homocisteína sobre TRAP, e a atividade da Na+,K+-ATPase e da CAT, indicando que o estresse oxidativo está provavelmente envolvido nos efeitos relacionados à homocisteína. Além disso, a administração crônica de homocisteína mostrou aumento de dano no DNA e prejuízo nas defesas antioxidantes (enzimáticas e não enzimáticas) em córtex parietal e plasma em um modelo experimental de hiperhomocisteinemia em ratos, nos quais os níveis plasmáticos de homocisteína atingiram valores similares aos encontrados em

humanos (Streck *et al.*, 2002; Matté *et al.*, 2009). É importante observar que nesses estudos os níveis de inibição enzimática assim como os efeitos sobre as defesas antioxidantes variaram entre 20 e 40% de inibição, comparável aos efeitos da homocisteína sobre a hidrólise de ATP e ADP mostrados nesse estudo, mesmo quando a concentração de 500 μM de homocisteína foi testada nos experimentos *in vitro*. Então, é plausível sugerir que a homocisteína induz o estresse oxidativo que é concentração limitada e a inibição observada deve ser devido ao número limitado de alvos redox em plaquetas e especialmente nas enzimas responsáveis pela hidrólise do ATP e do ADP na superfície da membrana das mesmas.

Dados da literatura indicam que a E-NTPDase1(ecto-apyrase) é a enzima responsável pela hidrólise de ATP e ADP na membrana de plaquetas (Frassetto *et al.*, 1993; Koziak *et al.*, 1999; Atkinson *et al.*, 2006; Duarte *et al.*, 2007; Lunkes *et al.*, 2009) . Entretanto, a presença de outras E-NTPDases não pode ser completamente excluída considerando que o efeito *in vitro* da homocisteína foi diferente sobre as atividades ATPásicas e ADPásicas. Neste sentido, considerando que as E-NTPDases são proteínas integrais de membrana, com dois domínios transmembrana, os quais são importantes para a atividade enzimática (Wang *et al.*, 1998; Robson *et al.*, 2006), é plausível sugerir um efeito indireto da homocisteína através de um desequilíbrio no estado redox das plaquetas, os quais devem alterar a função normal destas enzimas nas plaquetas, quando essas são expostas a homocisteína. Esta hipótese foi reforçada pelos resultados com a apirase purificada, visto que a homocisteína não afetou a hidrólise de ATP e ADP, reforçando o envolvimento da membrana da plaqueta na inibição da hidrólise causada pela homocisteina.

É bem estabelecido na literatura que a homocisteína se auto-oxida no plasma gerando EROs (Coppola *et al.*, 2000; Daví *et al.*, 2001; Jakubowski, 2006) e é capaz

também de induzir o estresse oxidativo em plaquetas (Leoncini *et al.*, 2007). Um estudo apontou que os radicais de oxigênio estão envolvidos na diminuição da hidrólise de ATP e ADP sobre a plaqueta e sugeriram um dano oxidativo na E-NTPDase1 de plaquetas para justificar a sensibilidade aumentada ao ânion superóxido na agregação plaquetária induzida por ADP quando comparado às condições de controle (Krötz *et al.*, 2002). Além do mais, a inativação da E-NTPDase1 por ânion superóxido, resultando em aumento na concentração de ADP, foi demonstrado por Robson e colaboradores (1997) em células endoteliais. Nos experimentos *in vivo* nos mostramos que o pré-tratamento dos animais com Vitamina E, um antioxidante lipídio-solúvel que atua como defesa contra o estresse oxidativo preveniu a inibição da hidrólise de ATP e ADP causada pela administração de homocisteína (Item 3.3, Figura 4, 5). Esses dados corroboram a hipótese de que o desequilíbrio do estado oxidativo está relacionado ao efeito inibitório da homocisteina sobre a atividade ATP/ADPásica.

Consistente com estudos prévios (Luo *et al.*, 2006; Mohan *et al.*, 2008), nós não encontramos nenhuma alteração quando somente a homocisteína foi adicionada ao plasma rico em plaqueta (PRP) (dados não mostrados), demonstrando que não houve agregação plaquetária espontânea somente com a presença da homocisteína. Por outro lado, trabalhos têm constatado um aumento na sensibilidade das plaquetas ocasionado pela homocisteína frente à agonistas em modelos animais (Durand *et al.*, 1996;1997), como observado em nossos resultados (Item 3.3, Figura 5). Portanto, nós sugerimos que a inibição da hidrólise de ADP após exposição à homocisteína poderia resultar em um aumento na reatividade e na agregabilidade de plaquetas, com um acúmulo de ADP no meio extracelular quando esse agonista é adicionado às plaquetas (Item 3.3, Figura 5). De outra forma, o ATP, um inibidor competitivo do ADP (Fijnheer *et al.*, 1992) sobre a agregação plaquetária, pode também acumular após a inibição da E-NTPDase1 e, pelo

menos em parte, poderia estar limitando agregações plaquetárias espontâneas. Além disto, durante injúria vascular, altos níveis de substâncias pró-agregantes tais como ADP, são liberados para o plasma (Sévigny *et al.,* 2002), o qual poderia desencadear a formação de trombos em estados patológicos. Então, neste cenário a inibição da E-NTPDase1, em conseqüência à exposição à homocisteína, resultaria em um aumento da resposta ao ADP e nessa linha reapresentaria um risco adicional para a trombogênese associada ao alto nível de homocisteína.

Com base nesses resultados, nós propusemos que a inibição da E-NTPDase1 causada pela homocisteína foi provavelmente devido ao estresse oxidativo, visto que antioxidantes preveniram tais efeitos. Em conjunto, esses achados devem contribuir para o desenvolvimento do risco trombótico atribuído a hiperhomocisteinemia e no desenvolvimento de estratégias terapêuticas associadas ao tratamento dessa patologia.

5. CONCLUSÕES

5.1 Gerais

- ✓ As ectonucleotidases apresentaram mudanças no padrão de expressão durante a diferenciação de fenótipos em macrófagos.

- ✓ A homocisteína alterou o perfil de ativação dos macrófagos e aumentou a hidrólise de ATP, ADP e AMP.

- ✓ Nas plaquetas a homocisteína causou uma diminuição nas hidrolises de ATP e ADP enquanto AMP não foi alterada.

5.2 Específicas

- ✓ A alterações no padrão de expressão das ENTPDase1, ENTPDase3 e ecto-5'-nucleotidase parecem ser uma característica na diferenciação fenotípica de macrófagos.

- ✓ As expressões dos receptores purinégicos foram similares nos fenótipos analisados, como demonstrado por RT-PCR, sugerindo que a atividade das ectonucleotidades deve controlar a sinalização purinérgica durante a ativação de macrófagos.

- ✓ Em macrófagos a homocisteína induz um perfil pró-inflamatório e um aumento na hidrólise do ATP, ADP e AMP com fortes evidências do envolvimento da E-NTPDase3 e da ecto-5'-nucleotidase nas mudanças inflamatórias associadas a hiperhomocisteinemia.

- ✓ Em macrófagos o tratamento com homocísteina mostrou um aumento da distribuição de ENTPDase3 na membrana das células enquanto a ecto-5`-nucleotidase não sofreu alteração em nível de RNA mensageiro nem no conteúdo protéico, sugerindo o envolvimento do estresse oxidativo.

- ✓ A origem e o tipo de estímulo podem e devem estar relacionado às mudanças de atividade e expressão das ectonucleotidases avaliadas, uma vez que macrófagos com o mesmo perfil de ativação (LPS e homocisteína) apresentaram um padrão de hidrólise diferentes.

- ✓ Em plaquetas a diminuição na hidrólise de ATP e ADP, provavelmente devido a inibição da E-NTPDase1, causada pela homocisteína revelou um envolvimento do estresse oxidativo, visto que antioxidantes preveniram tais efeitos.

- ✓ Em plaquetas a homocisteina causou um aumento na agregação plaquetária quando essa foi estimulada com ADP em comparação ao controle, sugerindo o envolvimento do sistema purinérgico no aumento do risco trombótico atribuído a hiperhomocisteinemia.

6. PERSPECTIVAS

Como continuação dessa Tese, pretende-se focalizar os seguintes objetivos:

- Estudar o efeito do dipiridamole, um inibidor do transportador de adenosina, sobre a produção de nitrito, TNF-α e IL-10 e avaliar o impacto disso na ativação de macrófagos pela homocisteína.
- Realizar estudos comparativos sobre o perfil de expressão das ectonucleotidases e dos receptores purinérgicos quando ativados por outro compostos com o mesmo padrão de ativação como por exemplo o ácido lipoteicóico (LTA), molécula de bactéria gram-positiva.
- Estudar comparativamente o perfil de expressão das ectonucleotidases e dos receptores quando submetidos a inversão do fenótipo, ou seja, os estímulos inicias, como por exemplo IL-4 e LPS serão invertidos após um determinado tempo: LPS sobre IL-4 (LPS/IL-4) e IL-4 sobre LPS (IL-4/LPS).
- Verificar o efeito dessas mudanças de expressão das ectonucleotidases, durante a ativação fenotípica de macrófagos, sobre a secreção de citocinas via receptores purinérgicos.
- Avaliar o perfil de outros importantes nucleotídeos, UTP e UDP, bem como seu envolvimento em processos fagocíticos envolvendo o receptor P2Y6.
- Estudar o efeito dos ácidos graxos saturados e poliinstaturados, como realizado com homocisteína, na ativação de macrófagos e na expressão das ectonucleotidases e dos receptores purinérgicos bem como o envolvimento do sistema purinérgico na secreção de citocinas inflamatória.

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8. ANEXOS

8.1 OUTROS ARTIGOS CIENTÍFICOS REALIZADOS EM CO-AUTORIA DURANTE O PERÍODO DO DOUTORADO

1. Braganhol, E, **Zanin RF,** Bernardi A, Bergamin LS. Cappellari A, Campesato LF, Morrone F, Campos M, Calixto, J, Edelweiss MI, Wink M, Robson S, Battastini AMO. Overexpression of CD39L1/NTPDdase2 in gliomas promotes systemic inflammation and pulmonary injury.
   Manuscrito em preparação

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