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EFEITOS DA HOMOCISTEÍNA E DO TRATAMENTO IMUNOSSUPRESSOR

COM CICLOSPORINA SOBRE A HIDRÓLISE DE NUCLEOTÍDEOS

EM SORO DE RATOS

ANA ELISA BÖHMER

Orientador:

Prof. Dr. DIOGO SOUZA

Co- Orientador:

PROF. Dr. JOÃO JOSÉ FREITAS SARKIS

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"Não é porque as coisas são difíceis que nós não ousamos.
É porque não ousamos que as coisas tornam-se difíceis."

Sêneca – filósofo romano, século I.

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SUMÁRIO

RESUMO

Durante os últimos anos, o aumento dos níveis circulantes de homocisteína (Hcy) passou a ser considerado um fator de risco independente para doenças cardiovasculares como aterosclerose, doenças vasculares periféricas, infarto do miocárdio e tromboembolismo. Essas complicações vasculares também podem estar relacionadas com os níveis extracelulares de nucleotídeos/adenosina, que modulam processos de agregação plaquetária, vasodilatação, fluxo sangüíneo coronariano e inflamação. Há estudos que sugerem uma relação entre Hcy e concentração de adenosina circulante. A hidrólise seqüencial de ATP até adenosina por ação de nucleotidases solúveis constitui um dos sistemas de degradação de nucleotídeos de adenina na circulação. Além disso, a ciclosporina (CsA), um potente imunossupressor que tem sido usado por pacientes transplantados, está associado a vários efeitos adversos. As doenças vasculares são a principal causa de morte entre pacientes transplantados, mas a relação da CsA com estas injúrias não está bem compreendida. Desta forma, objetivamos avaliar a participação da Hcy na modulação da hidrólise de nucleotídeos extracelulares de adenina em soro de ratos e ao mesmo tempo verificar os efeitos da administração de CsA sobre os níveis de Hcy total (tHcy), hidrólise de nucleotídeos de adenina e sua possível associação com doenças cardiovasculares em ratos. Nossos resultados demonstram que a Hcy inibibe a hidrólise de ATP, ADP e AMP em soro de ratos, *in vitro*, e as análises cinéticas indicam que esta inibição ocorre de forma acompetitiva. Também foi possível observar que ratos tratados com CsA apresentam um aumento estatístico significativo dos níveis de fibrinogênio, número de plaquetas e concentração de tHcy porém, a CsA induziu a diminuição da hidrólise de ATP, ADP e AMP e níveis de ácido úrico. A inibição da hidrólise dos nucleotídeos correlacionou negativamente com os níveis de tHcy e positivamente com os níveis de ácido úrico. Com este estudo demonstramos que o tratamento imunossupressor com CsA promove disfunções vasculares, já que favorece a formação de um estado pró-trombótico aumentando os níveis de plaquetas e fibrinogênio. Além disso, a CsA aumenta os níveis de tHcy e inibe a hidrólise de ATP, ADP e AMP, provavelmente diminuindo os níveis de adenosina circulante e seus efeitos protetores ao sistema cardiovascular. A forte correlação inversa entre níveis de tHcy e hidrólise de nucleotídeos apóiam nossos resultados *in vitro* e sugerem que a inibição da atividade das nucleotidases em ratos tratados com Csa seja dependente da Hcy. Os níveis diminuídos de ácido úrico circulante apontam uma relevância *in vivo* da inibição da hidrólise de nucleotídeos em soro. Contudo, a CsA favorece o surgimento de complicações vasculares através do aumento do número de plaquetas, dos níveis de fibrinogênio e tHcy, que por sua vez altera a hidrólise dos nucleotídeos de adenina em soro, compostos envolvidos na homeostasia cardiovascular.

ABSTRACT

During the past few years, elevated blood levels of homocysteine (Hcy) have been considered an independent risk factor for cardiovascular disease such as atherosclerosis, peripheral vascular disease, myocardial infarction and venous thromboembolism. These vascular complications can be also related to the ratio adenine nucleotide/adenosine, since extracellular these nucleotides are associated with modulation of processes such as platelet aggregation, vasodilatation, coronary blood flow and inflammation. Furthermore, there are some studies that suggest a relationship between Hcy and plasma adenosine concentrations. The sequential hydrolysis of ATP to adenosine by soluble nucleotidases constitutes one of the systems for rapid inactivation of circulating adenine nucleotides. Moreover, Cyclosporine (CsA), a potent immunosuppressant agent that has been extensively used in transplanted patients, is related to a variety of side effects. Vascular disease is a major cause of morbidity and mortality among transplant recipients, but the underlying mechanisms of vascular injury caused by cyclosporine are poorly understood. Thus, the main objective of this study was to evaluate if Hcy can participate in the modulation of the extracellular adenine nucleotide hydrolysis by rat blood serum and we also examined the effects of long-term CsA administration on total homocysteine (tHcy) levels, adenine-nucleotides hydrolysis, and its putative association with vascular disease in rats. Our results showed that Hcy inhibits *in vitro* ATP, ADP and AMP hydrolysis in rat blood serum and kinetic analysis showed that these inhibitions are of the uncompetitive type. At the same time, we observed that CsA induced a statistically significant increase in fibrinogen levels, platelets number and tHcy concentration, whereas induced a decrease in ATP, ADP and AMP hydrolysis and uric acid levels. The inhibition of nucleotides hydrolysis correlated negatively with total homocysteine levels and positively with uric acid levels. Here, we demonstrate that CsA long-term treatment induces vascular disturbances, since it might create a favorable scenario for a pro-thrombotic state, increasing platelets and fibrinogen levels. Additionally, it increases tHcy serum concentrations and inhibits serum ATP, ADP and AMP hydrolysis, probably decreasing serum adenosine levels and therefore its beneficial effects on the cardiovascular system. In support to our *in vitro* studies, the strong inverse correlation between tHcy levels and adenine nucleotides hydrolysis suggests that the inhibition of nucleotidase activities could be Hcy dependent. Low levels of uric acid during CsA treatment point that the inhibition of nucleotide hydrolysis might have *in vivo* relevance. In summary, CsA might create a favorable scenario for vascular complications by increasing platelets, fibrinogen and serum levels of tHcy, which in turn affects the hydrolysis of serum adenine nucleotides, compounds known to be involved in cardiovascular haemostasis.

LISTA DE ABREVIATURAS

ADP – adenosina 5'-difosfato

AMP – adenosina 5'-monofosfato

ATP – adenosina 5'-trifosfato

CBS – cistationina beta-sintase

CD39 – antígeno de ativação celular linfóide

CsA –ciclosporina

Hcy – homocisteína

Hhcy – hiperhomocisteinemia

MTHF – 5-metiltetraidrofolato

NTPDase – ecto-nucleotídeo trifosfato difosfoidrolase

SAH – S-adenosilhomocisteína

SAM – S-adenosilmetionina

tHcy –homocisteína total

1. INTRODUÇÃO

1.1 HOMOCISTEÍNA

Homocisteína (Hcy) é um aminoácido não-essencial formado a partir da desmetilação de metionina, um aminoácido especialmente abundante em carnes e laticínios. A Hcy está presente no sangue sob a forma oxidada (homocistina e mistura de dissulfetos) e sob a forma reduzida (tiol livre) (BUTZ & DU VIGNEAUD, 1932; UELAND, 1995). Por isso, o termo homocisteína total (tHcy) tem sido usado para definir o somatório desses diferentes tipos de Hcy encontrados em indivíduos saudáveis e, em maior concentração, em pacientes com hiperhomocisteinemia (Hhcy) (WELCH & LOSCALZO, 1998).

1.1.1 Metabolismo

O metabolismo da Hcy implica em duas vias metabólicas intracelulares: remetilação e transsulfuração (Anexo 1). No processo de remetilação, a Hcy recebe um grupamento metila a partir do 5-metiltetraidrofolato (CH_3THF) ou da betaína, dando origem ao aminoácido metionina. A reação com CH_3THF ocorre em todos os tecidos e é dependente de vitamina B12, enquanto a reação com a betaína ocorre principalmente no fígado e é independente da vitamina B12. A metionina pode então ser ativada pela adenosina trifosfato (ATP) e formar S-adenosilmetionina (SAM), um dos principais responsáveis pela transmetilação de ácidos nucléicos, neurotransmissores, fosfolipídeos e hormônios (HOFFER, 2004). A desmetilação de SAM gera S-adenosilhomocisteína (SAH), que ao ser hidrolisada produz Hcy e adenosina. A Hcy, por sua vez, pode ser remetilada, gerando a metionina e reiniciando o ciclo, ou pode seguir a via de transsulfuração.

No processo de transsulfuração a Hcy condensa-se a serina e produz cistationina, em uma reação irreversível e catalisada pela cistationina beta-sintase (CBS), e então é hidrolisada pela gama-cistationase gerando cisteína e alfa-cetobutirato (D'ANGELO & SELHUB 1997).

A Hcy intracelular é transportada para o sangue através de um mecanismo de exportação, que ajuda a manter o nível intracelular de Hcy baixo (UELAND et al., 1986; CHRISTENSEN et al., 1991). Quando o metabolismo da Hcy está alterado, o mecanismo de exportação limita a toxicidade intracelular porém, expõe o tecido vascular a altas concentrações de Hcy (D'ANGELO & SELHUB, 1997), ocasionando a chamada Hhcy. Em indivíduos saudáveis, a concentração de tHcy no sangue varia entre 5 e 15 µmol/L (JACOBSEN et al., 1994; UELAND et al., 1995) mas pode ser superior a 100 µmol/L em pessoas com Hhcy (KANG, WONG & MALINOW, 1992). Já pacientes com homocistinúria, erro inato do metabolismo da Hcy causado pela deficiência severa da enzima CBS, os níveis plasmáticos de tHcy podem ultrapassar 400 µmol/L (MUDD, LEVY & SKOVBY, 1995).

1.1.2 Homocisteína e doenças vasculares

Em 1969, McCully (MCCULLY, 1969) descreveu os problemas vasculares periféricos em pacientes com homocistinúria, revelando a importância da Hhcy severa no desenvolvimento da aterosclerose e do tromboembolismo. Desde então, observou-se que a concentração de tHcy é consistentemente mais elevada em pacientes com problemas vasculares periféricos, coronarianos e cerebrais quando comparado a pessoas normais (WELCH & LOSCALZO, 1998). Recentemente, estudos comprovaram que a Hhcy é um fator de risco

independente para aterosclerose (MALINOW, 1996), doenças vasculares periféricas (MAYER, JACOBSEN & ROBINSON, 1996; MINER, EVROVSKI & COLE, 1997), infarto do miocárdio (STUBBS et al, 2000) e tromboembolismo (BOUSHEY et al., 1995).

A Hcy parece alterar as propriedades anticoagulantes das células endoteliais para um fenótipo procoagulante (JACOBSEN, 1998; DEN HEIJER et al., 1998) e, além disso, altera a morfologia vascular, estimula a inflamação, danifica o endotélio e estimula as vias de coagulação (UPCHURCH et al, 1997; STANGER et al., 2004).

O aumento da tHcy está relacionado a diversos efeitos aterotrombogênicos porém, os mecanismos pelos quais a Hcy promove problemas vasculares ainda são pouco conhecidos. A diminuição da disponibilidade de óxido nítrico (vasodilatador endógeno), como conseqüência do aumento do estresse oxidativo causado pela Hcy, é sugerida como uma possível causa de disfunções endoteliais (FARACI, 2003). Harker et al. (HARKER et al., 1974) propõe que o dano endotelial causado pela Hcy seja mediado pelo peróxido de hidrogênio, que expõe a matriz do vaso e as células musculares lisas, fazendo-as proliferarem e promoverem a ativação plaquetária e leucocitária. Além disso, a produção de superóxido poderia ativar a peroxidação lipídica, da membrana do endotélio vascular e das lipoproteínas, propiciando condições favoráveis para a formação de placas ateromatosas (HUANG et al., 2001).

No entanto, além do aumento na produção de radicais livres, descrito anteriormente, estudos recentes têm relacionado os distúrbios vasculares causados pela Hcy com a diminuição dos níveis circulantes de adenosina (AGTERESCH, 1999 ; CHEN, LI & ZOU, 2002; RIKSEN et al., 2005), um

nucleosídeo amplamente conhecido por suas propriedades cardioprotetoras (BELARDINELLI, LINDEN & BERNE, 1989; RIKSEN et al., 2003).

1.2 SISTEMA CARDIOVASCULAR E SINALIZAÇÃO PURINÉRGICA

Nucleotídeos e nucleosídeos extracelulares exercem variadas respostas, mediadas por receptores, em diferentes tecidos (ABBRACCHIO & BURNSTOCK, 1998; FISCHER, 1999). No sistema nervoso central, os nucleotídeos purínicos têm sido envolvidos em muitos processos fisiológicos, incluindo transmissão sináptica, neuromodulação, neurogênese, apoptose e aprendizado (ZIMMERMANN, 1994; FREDHOLM et al., 1993; WEAVER, 1996; ABBRACCHIO et al., 1995; BONAN et al., 2000). No sistema cardiovascular as purinas atuam em processos de dilatação e contração vascular, agregação plaquetária, proliferação celular, resposta inflamatória e dor (RALEVIC & BURNSTOCK, 2003).

Os efeitos extracelulares das purinas são exercidos mediante interação com receptores do tipo P1 e P2, localizados na superfície da membrana celular. Os receptores P1, seletivos para adenosina, são divididos de acordo com suas propriedades moleculares, bioquímicas e farmacológicas: A_1, A_{2A}, A_{2B} e A_3, todos acoplados a proteínas-G. Já os receptores P2, seletivos para ATP, dividem-se em duas grandes famílias: P2X (subdividida em oito membros – $P2X_{1-8}$), ligado a um canal ionotrópico e envolvido na transmissão excitatória rápida; e P2Y (subdividida em $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$), ligado a uma proteína-G (ABBRACCHIO & BURNSTOCK, 1994; FREDHOLM et al., 1994; RALEVIC & BURNSTOCK, 2003).

Todas as células do sistema cardiovascular expressam um ou mais subtipos de receptores de purinas (RALEVIC & BURNSTOCK, 2003) e dependente da célula e do receptor as purinas podem desempenhar numerosos efeitos benéficos sobre o sistema cardiovascular. BERNE, 1963, demonstrou que o nucleosídeo adenosina é um mediador de vasodilatação e aumento do fluxo sangüíneo durante hipóxia. Desde então, vários estudos têm demonstrado que a adenosina e os nucleotídeos derivados da adenina estão envolvidos no controle de diversos processos biológicos que incluem efeitos importantes sobre o sistema cardiovascular.

O nucleotídeo ATP, extracelularmente, é capaz de exercer efeitos opostos no sistema vascular. Quando liberado pelos nervos simpáticos, o ATP atua sobre os receptores P2X da musculatura vascular lisa como um agente vasoconstritor, enquanto durante hipóxia o ATP é liberado pelas células endoteliais e atua sobre receptores P2Y das mesmas, induzindo a liberação de óxido nítrico, um agente vasodilatador (RALEVIC & BURNSTOCK, 2003).. O ATP também é liberado para a corrente sangüínea a partir de plaquetas e eritrócitos rompidos e ainda pode exercer outras funções no sistema cardiovascular como: controle da proliferação celular em células endoteliais e musculares lisas, arritmia, hipertrofia cardíaca, apoptose e regulação da reatividade plaquetária (RALEVIC & BURNSTOCK, 2003).

Na circulação, o nucleosídeo difosfatado ADP está relacionado com a regulação da ativação e recrutamento de plaquetas, sendo o principal promotor de agregação plaquetária mediante interação com receptores $P2Y_{12}$. Assim sendo, o controle dos níveis extracelulares de ADP é de grande importância para

a regulação dos processos tromboticos (BORN, 1962; BORN & CROSS, 1963; GACHET, 2001).

A adenosina, além de ser um potente vasodilatador, agindo nos receptores A_2 das células endoteliais (RALEVIC & BURNSTOCK, 2003), também inibe a agregação plaquetária via estimulação da adenilato ciclase pelos receptores A_{2A} plaquetários (BELARDINELLI, LINDEN & BERNE , 1989; CRISTALLI, et al., 1994); inibe a proliferação das células musculares lisas e endoteliais (DUBEY, 1997; BURNSTOCK, 2002) e a adesão de neutrófilos ao endotélio vascular (CRONSTEIN, 1996). Desta forma, a adenosina desempenha um papel protetor importante em processos de isquemia, hipóxia, hipertensão, aterosclerose, trombose e inflamação (MUBAGWA et al., 1996; RALEVIC & BURNSTOCK, 2003).

A adenosina circulante tem duas origens distintas: liberação celular para a corrente sangüínea via transportadores bidirecionais de nucleosídeos ou então pode ser produzida pela degradação dos nucleotídeos extracelulares ATP, ADP e AMP (LATINI & PEDATA, 2001). A manutenção da sinalização purinérgica normal depende da concentração destes nucleotídeos/nucleosídeo circulantes, e este controle ocorre principalmente através da atividade de enzimas nucleotidases, responsáveis pela hidrólise de nucleotídeos da adenina, entre outros.

1.3   HOMOCÍSTEINA E ADENOSINA

Estudos têm demonstrado que pacientes com Hhcy apresentam baixos níveis de adenosina tecidual e circulante e atribuem à diminuição de adenosina os problemas cardiovasculares destes pacientes (CHEN, LI & ZOU, 2002).

Por desempenhar um papel importante no controle da homeostasia cardiovascular (BELARDINELLI, LINDEN & BERNE, 1989; DUBEY, 1997; RALEVIC & BURNSTOCK, 2003), a diminuição de adenosina circulante tem sido relacionada a vasoconstrição, aterosclerose, trombose e outras complicações cardiovasculares semelhantes àquelas observadas em pacientes com Hhcy. Riksen et al. (2005) também propõe que a diminuição de adenosina causada por elevados níveis de tHcy seria uma das alterações que contribuem para o desenvolvimento das doenças cardiovasculares

Pouco se sabe a respeito dos mecanismos que induzem a diminuição dos níveis de adenosina circulante durante a Hhcy. No entanto, alguns pesquisadores sugerem que a diminuição nas concentrações de adenosina intracelular esteja envolvida (DEUSSEN, 1999; RIKSEN et al., 2003). Em condições normais, a enzima S-adenosilhomocisteína-hidrolase (Anexo 2) hidrolisa SAH até Hcy e adenosina mas, na presença de altas concentrações de Hcy intracelular, apresenta atividade reversa e utiliza adenosina para produzir SAH (RIKSEN et al., 2003; RIKSEN et al., 2005). Nestas circunstâncias os níveis intracelulares de adenosina diminuem e ao mesmo tempo aumenta o gradiente de concentração transmembrana de adenosina, conseqüentemente diminuindo os níveis de adenosina extracelular (DEUSSEN, 1999).

No entanto, como já relatado anteriormente, as concentrações plasmáticas de adenosina são determinadas tanto pela atividade celular dos transportadores bidirecionais de nucleosídeos como pela degradação extracelular dos nucleotídeos precursores de adenosina (LATINI & PEDATA, 2001). Desta forma, além da atividade intracelular da S-adenosilhomocisteína-hidrolase, a hidrólise de nucleotídeos de adenina (ATP, ADP e AMP) no meio extracelular   também

representa um importante mecanismo de produção de adenosina circulante (BORST & SCHRADER, 1991). Porém, os efeitos da Hcy sobre as enzimas nucleotidases que hidrolisam estes nucleotídeos extracelulares ainda não são bem estabelecidos.

1.4   NUCLEOTIDASES

Os nucleotídeos extracelulares podem ser hidrolisados por uma variedade de enzimas localizadas nas membranas celulares ou presentes na forma solúvel nos meios intra e extracelulares. Uma vez liberados no espaço extracelular, os nucleotídeos exercem diversos efeitos como moléculas sinalizadoras e seus efeitos são modulados pela ação das nucleotidases (ZIMMERMANN, 2001). Assim, as nucleotidases desempenham um importante papel no controle da concentração dos nucleotídeos e nucleosídeos extracelulares.

As nucleotidases são ecto-enzimas envolvidas no controle dos níveis dos nucleosídeos tri- e difosfatados e pertencem às famílias: E-NTPDases (ectonucleosídeo trifosfato difosfoidrolase), E-NPPs (ectonucleotídeo pirofosfato/ fosfodiesterase) e fosfatases alcalinas. Nucleosídeos monofosfatados estão sujeitos a ação da E-5'-nucleotidase porém, também podem ser hidrolisados pela fosfatase alcalina e por alguns membros da família das E-NPPs (ZIMMERMANN, 2001).

As E-NTPDases são enzimas que catalisam a hidrólise dos resíduos de fosfato gama e beta dos nucleotídeos. A grande família das E-NTPDases é composta por oito membros: NTPDase1, NTPDase2, NTPDase3, NTPDase4, NTPDase5, NTPDase6, NTPDase 7 e NTPDase 8 (Anexo 3). Estas enzimas parecem estar envolvidas nos processos fisiológicos de neurotransmissão,

função cardíaca, agregação plaquetária, adesão celular, tônus vascular, resposta imune e crescimento celular (ZIMMERMANN, 2001; SHI et al., 2001; BIGONNESSE et al., 2004).

As NTPDase1-3 são enzimas ancoradas na membrana celular pelos terminais N-terminal e C-terminal, apresentam uma extensa alça transmembrana e o sítio ativo voltado para o espaço extracelular. Estas NTPDases hidrolisam os nucleotídeos das purinas e pirimidinas, suas atividades catalíticas máximas estão adaptadas ao meio extracelular e requerem cátions divalentes como o Ca^{2+} ou Mg^{2+} e um pH alcalino. A principal diferença entre estas três enzimas (NTPDase1, 2 e 3) é a especificidade pelo substrato.

A NTPDase1 (CD39, ecto-apirase ou ecto-ATP difosfoidrolase) hidrolisa ATP e ADP igualmente bem, sendo 1:1 a proporção da hidrólise destes dois substratos (KACZMAREK et al., 1996; WANG & GUIDOTTI, 1996; ZIMMERMANN, 2001). A NTPDase2 (CD39L1, ecto-ATPase) tem uma preferência muito maior pelo ATP do que pelo ADP (30:1) como substrato, agindo como um produtor extracelular de ADP. A NTPDase3 (CD39L3) tem propriedades funcionais intermediárias, hidrolisando ATP aproximadamente três vezes mais que o ADP (1:3) (SMITH & KIRLEY, 1998). Sabe-se que estas três enzimas podem produzir diferentes impactos na sinalização purinérgica porém, suas especificidades e conseqüências específicas nas células ou tecidos ainda são pouco compreendidas (ZIMMERMANN, 2001).

A NTPDase4, embora possua a mesma estrutura geral das NTPDases1, 2 e 3, tem uma localização celular completamente diferente. Duas formas encontradas em humanos têm sido localizadas no complexo de Golgi (UDPase,

NTPDase4β) e em vacúolos lisossômicos e autofágicos (NTPDase4α) (WANG & GUIDOTTI, 1998).

A NTPDase5 e a NTPDase6 estão ancoradas na membrana celular somente pela porção N-terminal e por isso seriam facilmente liberadas para o meio extracelular. Estudos em murinos sugerem, que esta enzima está localizada no retículo endoplasmático e estudos com células COS-7 sugerem que esta seria uma forma secretada e, portanto solúvel. A NTPDase6 também é uma enzima secretada solúvel, porém a origem desta enzima seria no complexo de Golgi. Ambas as enzimas hidrolisam nucleosídeos difosfatados com uma alta preferência e além disso, acredita-se que elas participam nas reações de reglicosilação envolvidas nos processos de dobramento de glicoproteínas e em processos glicosilação no complexo de Golgi (ZIMMERMANN, 2001).

A NTPDase7 e NTPDase8 foram recentemente descritas. A NTPdase7 assemelha-se as NTPDases intracelulares (NTPDase4-7) (SHI et al., 2001) enquanto a NTPDase8 possui características similares as NTPDases1-3 (BIGONNESSE, et al., 2004).

A 5'-nucleotidase (CD73) (Anexo 3) é uma enzima amplamente distribuída em bactérias, células vegetais e em tecidos de vertebrados. Esta enzima está classificada em quatro grupos, de acordo com a localização celular e as propriedades bioquímicas: ecto-5'-nucleotidase é ancorada a membrana plasmática; uma forma solúvel e derivada da ecto-5'-nucleotidase; e duas formas citoplasmáticas. Estas enzimas controlam os níveis intra e extracelulares de AMP e outros nucleosídeos monofosfatados (ZIMMERMANN, 1992; KAWASHIMA, NAGASAWA & NINOMIYA, 2000).

As NTPDases podem produzir efeitos através da atividade conjunta com a 5'-nucleotidase, hidrolisando os nucleosídeos fosfatados até os respectivos nucleosídeos. A associação destas enzimas é capaz de controlar a concentração de nucleotídeos e nucleosídeos extracelulares e portanto, modular processos fundamentais a nível celular em muitos tecidos e órgãos, principalmente no sistema cardiovascular. Portanto, a atividades das nucleotidases que hidrolisam nucleotídeos extracelulares associadas à ação da 5'-nucleotidase são importantes agentes terapêuticos contra doenças vasculares pela formação da adenosina circulante (Gayle et al., 1998).

1.5   CICLOSPORINA

Ciclosporina (CsA) é uma droga imunossupressora potente e efetiva, amplamente utilizada no combate à rejeição de órgãos transplantados e em doenças auto-imunes (MERION, WHITE & THIRU, 1984; COLE et al., 1998; VERCAUTEREN et al., 1998). Trata-se de um polipeptídeo cíclico de 11 resíduos de aminoácidos, extraído do fungo *Tolypocladium inflatum gams*, que atua como agente inibidor de calcineurina.

A ação imunossupressora da CsA ocorre a partir da formação de um complexo com seu receptor citoplasmático, a ciclofilina, que inibe a calcineurina fosfatase. Isto implica na inibição do fator nuclear de ativação de células T (NFAT) e conseqüente interrupção da transcrição dos genes da interleucina-2, interferon-gama, TNF-alfa e outras citocinas indispensáveis para a ativação da resposta imune (SCHREIBER & CRABTREE, 1992; SHIBASAKI, HALLIN & UCHINO, 2002). Assim, a CsA é conhecida como um agente imunossupressor

inibidor de calcineurina, que atua inibindo a produção de interleucinas pelas células T do sistema imune.

1.5.1 *Ciclosporina e doenças vasculares*

O uso terapêutico da CsA pode ser acompanhado por alguns efeitos adversos como nefrotoxicidade, neurotoxicidade, dislipidemia, hipertensão, vasoconstrição e doenças vasculares obstrutivas (KAHAN, 1989; WEIS & VON SCHEIDT, 1997; VAZIRI, LIANG & AZAD, 2000, SERKOVA, CHRISTIANS & BENET LZ, 2004).

A doença arterial coronariana está presente na maioria dos pacientes transplantados de coração (MCALLISTER, RADOVANCEVIC & FRAZIER, 1996) e eventos ateroscleróticos freqüentemente se manifestam por lesão do epitélio vascular do órgão transplantado (HOSENPUD, SHIPLEY & WAGNER, 1992; FUJITA, 1993). Ao mesmo tempo, há evidências de que o uso prolongado de CsA esteja associado a doenças vasculares obstrutivas progressivas (HOSENPUD, SHIPLEY & WAGNER, 1992; WEIS & VON SCHEIDT, 1997). Estas vasculopatias são as principais causas de morte entre pacientes transplantados, superando até mesmo a infecção (BECKER et al., 1988; DEMIRAG, et al.,1998).

Pouco se sabe sobre os mecanismos de ação da CsA nesses incidentes cardiovasculares, uma vez que a presença do órgão transplantado e a co-administração de outros medicamentos dificulta essa investigação. No entanto, alguns estudos demonstram que a administração de CsA está associada ao aumento das concentrações de tHcy em pacientes transplantados (AMBROSI et

al., 1994; ARNADOTTIR et al., 1996; ARNADOTTIR et al., 1998; COLE et al., 1998; GUPTA et al., 1998; HERRERO et al., 2000). Conforme relatado anteriormente, o aumento da tHcy é um fator de risco independente para aterosclerose (MALINOW, 1996), doenças vasculares periféricas (MAYER, JACOBSEN & ROBINSON, 1996; MINER, EVROVSKI & COLE, 1997), infarto do miocárdio (STUBBS et al, 2000) e tromboembolismo (BOUSHEY et al., 1995).

Além disso, há estudos indicando que a atividade nucleotidásica vascular está comprometida em pacientes submetidos ao transplante (KOYAMADA et al., 1996; IMAI et al.,1999; ROBSON et al., 1999; LI et al., 2003). Porém, o efeito da CsA sobre a atividade de hidrólise de nucleotídeos extracelulares não está estabelecido.

1.6 OBJETIVOS

1.6.1 Objetivo geral

Considerando as complicações vasculares associadas à administração da CsA, entendemos ser importante investigar a sua possível relação com a concentração plasmática de tHcy e a atividade das nucleotidases. Isto poderia auxiliar na determinação de potenciais fatores envolvidos nos processos de vasculopatias presentes em pacientes que fazem uso da terapia imunossupressora.

1.6.1 Objetivos específicos

- Avaliar o efeito *in vitro* da Hcy nas atividades nucleotidásicas de hidrólise de ATP, ADP e AMP em soro de ratos;
- Investigar o efeito do tratamento imunossupressor com CsA nas concentrações de tHcy em soro de ratos não transplantados;
- Avaliar os efeitos do tratamento imunossupressor com CsA na atividade nucleotidásica de hidrólise ATP, ADP e AMP em soro de ratos não transplantados;
- Investigar os efeitos do tratamento imunossupressor com CsA sobre alguns parâmetros de coagulação.

2. ARTIGOS

2.1 CAPÍTULO 1

IN VITRO EFFECT OF HOMOCYSTEINE ON NUCLEOTIDE HYDROLYSIS

BY BLOOD SERUM FROM ADULT RATS

Em fase de publicação pela revista Chemico-Biologial Interactions

IN VITRO EFFECT OF HOMOCYSTEINE ON NUCLEOTIDE HYDROLYSIS BY BLOOD SERUM FROM ADULT RATS.

Ana Elisa Böhmer, Daniela Pochmann and João J.F. Sarkis.

 Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde,

Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600- Anexo

CEP 90035-003, Porto Alegre, RS, Brazil.

* Address correspondence to:

Dr. João José de Freitas Sarkis

Universidade Federal do Rio Grande do Sul

Departamento de Bioquimica, ICBS

Rua Ramiro Barcellos, 2600-ANEXO

CEP 90035-003, Porto Alegre, RS, Brazil

Phone number: +55 (51) 33165554

Fax number: +55 (51) 33165540

e-mail address: jjsarkis@plugin.com.br

Abstract

During the past few years, elevated blood levels of homocysteine (Hcy) have been linked to increased risk of premature coronary artery disease, stroke and thromboembolism. These processes can be also related to the ratio adenine nucleotide/adenosine, since extracellularly these nucleotides are associated with modulation of processes such as platelet aggregation, vasodilatation and coronary flow. Furthermore, there are some studies that suggest a relationship between Hcy and plasma adenosine concentrations. The sequential hydrolysis of ATP to adenosine by soluble nucleotidases constitutes one of the systems for rapid inactivation of circulating adenine nucleotides. Thus, the main objective of this study was to evaluate if Hcy can participate in the modulation of the extracellular adenine nucleotide hydrolysis by rat blood serum. Our results showed that Hcy, at final concentrations of 5.0 mM, inhibits *in vitro* ATP, ADP and AMP hydrolysis by 26%, 21% and 16%, respectively. Also Hcy, at final concentrations of 8.0 mM, inhibited the *in vitro* hydrolysis of ATP, ADP and AMP by 46%, 44% and 44%, respectively. Kinetic analysis showed that the inhibitions of the three adenine nucleotide hydrolyses in the presence of Hcy, by serum of adult rats, is of the uncompetitive type. The IC50 calculated from the results obtained were 6.52 ± 1.75 mM (n= 4), 5.18 ± 0.64 mM (n= 3) and 5.16 ± 1.22 mM (n= 3) for ATP, ADP and AMP hydrolysis, respectively.

Key words: nucleotidases, blood serum, homocysteine, cardiovascular disease

1. Introduction

Homocystinuria, the most common inherited disorder of sulfur amino acid metabolism, is caused by severe deficiency of cystathione β-synthase activity (CBS-deficiency) and is biochemically characterized by tissue accumulation of homocysteine (Hcy) [1]. Affected patients present alterations in various organs and systems, particularly in the central nervous system and in the vascular system [2].

Homocystinuric patients commonly have thromboembolic complications due to accelerated atherosclerosis caused by vascular wall injury through free radicals and by increased platelet adhesiveness that is secondary to high plasma Hcy levels [2,3]. At the same time, hyperhomocysteinemia (Hhcy) has been increasingly recognized as an independent risk factor for atherosclerosis [3], peripheral vascular disease [4], coronary heart disease [5], myocardial infarction [6-8], cerebrovascular disease [9] and venous thromboembolism [10]. However, mechanisms of action remain poorly understood.

Many studies have demonstrated *in vitro* effects of Hcy that may be relevant to atherogenesis and trombogenesis, such as: induces oxidative stress; induces endothelial dysfunction as a result of increased oxidative stress; enhances concentration of asymmetrical dimethylarginine (ADMA); decreases bioavailability of nitric oxide (due to increased oxidative stress), and increases inflammation; induces hypertrophy and altered mechanics in the microcirculation; increases intima media thickness; increases platelet aggregation; enhances binding of lipoprotein to fibrin and interferes with several clotting factors [11-14]. In support of these findings, *in vitro* studies have also demonstrated that Hcy

enhances the production of several pro-inflammatory cytokines [15-17] and increases the expression of monocyte chemoattractant protein 1 (MCP-1), involved in the pathogenesis of atherosclerosis by promoting recruitment of inflammatory cells to the vessel wall, in cultured human vascular endothelial cells, smooth muscle cells and monocytes [18-20]. There are also some studies suggesting that Hcy decreases plasma and tissue adenosine (Ado) concentrations [21,22]. This decrease in adenosine could well contribute to the cardiovascular complications of Hhcy.

Extracellular ATP and its breakdown products, ADP and adenosine, have been shown to present pronounced effects on a variety of biological and pathological processes [23,24]. ATP has been suggested to play a role in vascular tone, cardiac function and renal epithelial transport [25], and it was observed that micromolar concentrations of ATP inhibit platelet aggregation by both competitive and non-competitive mechanisms; however, low concentrations are stimulatory [26]. ADP is a nucleotide known to induce changes in platelet shape and aggregation. Several authors have described the important role of these nucleotides in the process of haemostasis and thrombus formation [27-30]. The nucleoside adenosine produced by nucleotide degradation, at the same time that inhibits platelet aggregation, is a structure capable of acting as a vasodilator, an important effect to consider adenosine a cardioprotector.

Extracellular nucleotides concentration can be regulated by the action of ecto- and soluble nucleotidases, including enzymes of the E-NTPDase family as well as the 5'-nucleotidase enzyme [31]. Ecto-ATP diphosphohydrolase (EC 3.6.1.5, NTPDase, ecto-apyrase, CD39) is a member of NTPDase family that hydrolyzes ATP, ADP and other triphospho- and diphosphonucleosides to their

equivalent monophosphonucleoside (AMP) and inorganic phosphate [31,32]. Over the last few years our group has demonstrated a soluble NTPDase activity in rat blood serum [33]. This soluble nucleotidase acts together with 5'-nucleotidase (EC 3.1.3.5, CD73), which also participates in adenine nucleotides metabolism in the circulation hydrolyzing the monophosphonucleoside, AMP, to inorganic phosphate and adenosine. This enzymatic cascade control the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors, and consequently, the duration and extent of receptor activation [34]. Therefore, this cascade formed by soluble NTPDase (apyrase) and 5'nucleotidase is an enzymatic pathway with a double function of removing a signal of ATP and generating a second one, produced by adenosine.

The main objective of the present study was to evaluate the *in vitro* effects of Hcy upon the enzymes involved in nucleotide hydrolysis by blood serum obtained from adult male Wistar rats.

2. Materials and methods

2.1. Subjects and Reagents

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. Adult male Wistar rats of approximately 60 days old, weighting around 250 grams, were maintained under a standard dark-light cycle, at a room temperature of 22 + 2°C. The rats had free access to food and water. Animal care followed the official governmental guidelines in

compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. Nucleotides, Trizma Base and DL-Homocysteine were purchased from Sigma Chemical Co., St Louis, MO, USA. All others reagents were of analytical grade.

2.2. Isolation of blood serum fraction

Blood was drawn after decapitation of the male Wistar rats, as described by Oses et al. [33]. Blood samples were centrifuged in plastic tubes for 5 minutes at 5000 X g , 20oC and kept on ice until used. Serum samples were preincubated with Hcy or vehicle immediately.

2.3. Measurement of ATP and ADP hydrolysis

ATP and ADP hydrolyses were performed using the method described previously [33]. The reaction mixture containing ADP or ATP as substrate, 112.5 mM Tris-HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37°C for 40 minutes in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of 10% TCA. The samples were chilled on ice and the amount of inorganic phosphate (Pi) liberated was measured as previously outlined [35]. Incubation times and protein concentration were chosen to ensure the linearity of the reaction (results not shown). In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with TCA. All samples were centrifuged at 5000 g for 5 minutes to

eliminate precipitated protein and the supernatant was used for the colorimetric assay. All samples were assayed in duplicate. Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein. DL-Hcy was dissolved in Tris-HCl buffer, pH8.0, and preincubated for 24 hours at room temperature with rat serum.

2.4. Measurement of AMP hydrolysis

To evaluate the AMP hydrolysis, we used a reaction mixture containing AMP as substrate to a final concentration of 3.0 mM in 100mM Tris-HCl, pH 7.5, incubated with 1.0 mg of serum protein at $37^{o}C$ in a final volume of 0.2 mL. All other procedures were the same as described above for ATP and ADP hydrolysis.

2.5. Protein determination

Protein was measured by the Coomassie Blue method [36], using bovine serum albumin as standard.

2.6. Data analysis

Data were analyzed using one-way ANOVA, followed by the Tukey's multiple range test. $P < 0.05$ was considered to represent a significant difference in the statistical analysis used. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software. The inhibition type was characterized using the classical Lineweaver-Burk plot.

3.   Results

3.1. Inhibition of ATP, ADP and AMP hydrolysis

The *in vitro* effects of Hcy on adenine nucleotide hydrolysis are shown in Fig.1. ATP, ADP and AMP hydrolysis were significantly inhibited in the presence of 5.0 mM Hcy (26%, 21% and 16% respectively, $P<0,05$). This inhibition was concentration dependent (for the values tested) since the enzyme activity was markedly reduced in the presence of 8.0 mM Hcy (46%, 44%, and 44% respectively, $P<0.005$), when compared with control group. Besides differing from the control group, Hcy 8.0 mM group also was significantly different from Hcy 5.0 mM group ($P < 0.05$). These results were decisive to design the next set of experiments.

3.2. Kinetics of the homocysteine interaction with ATP, ADP and AMP hydrolysis

The kinetics of the interaction of Hcy with ATPase, ADPase and AMPase activities using adult rat blood serum were determined. The Lineweaver–Burk double-reciprocal plot was analyzed over a range of concentrations (0.2 – 0.4 mM) of ATP, ADP or AMP as substrates in the absence and presence of Hcy 5.0 mM and 8.0 mM (Fig. 2). The results obtained indicated that the type of inhibition is uncompetitive by Hcy for ATP, ADP (NTPDase) and AMP (5'-nucleotidase) hydrolysis by rat blood serum.

3.3. IC50 value determination

After the determination of the type of inhibition for ATP, ADP and AMP hydrolysis caused by Hcy, we determined the IC50 for the hydrolysis of each one of the substrates for both enzymes (NTPDase and 5'-nucleotidase) from rat blood serum. IC50 values were graphically determined (plot not shown) according to the Dixon and Webb method [37]. For the calculation of IC50, $1/V$ values were expressed in a plot against Hcy concentrations (5.0 mM and 8.0 mM). The IC50 calculated from the results were 6.52 ± 1.75 mM, 5.18 ± 0.64 mM and 5.16 ± 1.22 mM (mean $\pm$ S.D., n = 3) for ATP, ADP and AMP hydrolysis, respectively.

4.   Discussion

Compared with other organ systems, the cardiovascular system is particularly sensitive to elevated Hcy levels [38]. A moderate elevation in plasma concentration of Hcy may alter vascular morphology, stimulate inflammation, damage the endothelium and the blood-clotting cascade, inhibit fibrinolysis and decrease the availability of nitric oxide (due to increased oxidative stress) [39,40]. As a result of this set of events, Hhcy has been associated with loss of endothelial antithrombotic function and induction of a procoagulant environment [41], a very harmful condition for health.

Vascular disorders can be also associated to an unbalance in the ratio nucleotides/nucleoside in the circulation; however, the effects of Hcy on adenine nucleotides metabolism in serum had still not been investigated.

In the present study we observed decrease in ATP, ADP and AMP hydrolysis in rat blood serum after 24 hours of sample preincubation with Hcy. The kinetic analysis of Hcy upon ATP, ADP and AMP hydrolysis showed an uncompetitive inhibition (occurs when the inhibitor binds only to the enzyme-substrate complex). This type of inhibition frequently occurs with enzymes that have an ordered sequence of substrate binding, such as occurs with ATP diphosphohydrolase. Despite our results consider unphysiological Hcy concentration, in the milimolar range, and *in vitro* condition we need to consider that *in vivo* other factors could affect the enzymatic cascade (ATPase-ADPase-AMPase activities) and the nucleotide hydrolysis inhibition could be reached at lower Hcy concentrations than that used in our experiments. Though, this information might be relevant in designing medicines to avoid problems related to increased levels of Hcy in the circulation.

It is tentative to speculate that the effects exerted by Hcy upon nucleotide hydrolysis by rat blood serum may be part of a complex mechanism to promote an increase in ATP, ADP and AMP extracellular levels and at the same time a decrease in extracellular adenosine level. Our results are in agreement with Chen et al. [42], which demonstrated that deficiency of plasma adenosine levels might be one of the important mechanisms resulting in pathological changes in cardiovascular and other organ systems during Hhcy, since chronic elevations of plasma Hcy concentration resulted in a sustained low level of plasma adenosine in rats.

In the cardiovascular system, ATP and other nucleotides can be released by cell lyse and/or cell death as well as exocytosis. Their biological effects are mainly determined by their rate of release in the extracellular medium, the activity

of nucleotidases and their binding affinity to specific receptors. We need to consider that the modulation of nucleotidases will affect the nucleotide levels and by this way the cell signaling on nucleotide receptors in different types of cells. Inhibition of nucleotidases may prolong the effect of nucleotides ATP, ADP and AMP at their respective receptors [43,44].

Extracellularly, the nucleotide ATP has important vascular actions [45]. In elevated concentrations, it induces vasoconstriction of the vascular wall, and, at the same time, promotes its own stimulated release from endothelial cells. On the other hand, ADP is a potent platelet-recruiting factor inducing platelet aggregation via interaction of platelet $P2Y_{12}$ receptors [46]. Thus, the inhibition found for ATP and ADP hydrolysis by Hcy in blood serum could enhance the ATP-induced vasoconstriction [47] and the platelet aggregation mediated by ADP, the most potent platelet agonist [48]. In addition, inhibition of AMP hydrolysis may contribute to a decrease in adenosine levels, a molecule that has a number of cardiovascular protective effects such as vasodilatation and inhibition of platelet aggregation [49].

Studies have attempted to address the possible mechanisms by which elevated levels of Hcy may represent a risk factor for atherosclerosis, thrombosis, pulmonary embolism, cerebrovascular disease, increased peripheral vascular resistance and myocardial infartation. Considering our results, the seen modulation could contribute to the understanding of the mechanisms by which Hcy acts in the organism, since the resulting pathological symptoms of the diminished level of adenosine are similar to those during Hhcy. Moreover, although plasma concentrations of Hcy are partly genetically determined, acquired states such as folic acid and vitamin B12 deficiencies may also increase Hcy

levels [50-52].   Since folate intake is inversely related to circulating Hcy concentrations, it is interesting to think that folic acid might interact with Ecto-ATP diphosphohydrolase and 5'-nucleotidase, or either that folic acid could revert the inhibition effect of Hcy on ATP, ADP and AMP hydrolysis. Additional studies will be necessary to confirm this hypothesis, in terms of a possible therapeutical role.

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Legends and figures

Fig. 1. Effect of homocysteine on ATP, ADP and AMP hydrolysis by rat blood serum. Values are mean ± S.D. for four independent experiments. Results are expressed as specific activity (nmoles of phosphate/minute/mg protein), considering the values of controls as 100% (absolute value for control groups: 1.34 ± 0.14 for ATPase, 1.83 ± 0.16 for ADPase and 1.30 ± 0.18 for AMPase activity). * indicates significant difference from control group (P < 0.05) and # indicates significant diference from control and Hcy 5 mM group (P < 0.05). Data were analyzed statistically using one-way ANOVA, followed by the Tukey's multiple range test

Fig. 2. Kinetic analysis of the inhibition of ATP (A), ADP (B) and AMP (C) hydrolysis by homocysteine in rat blood serum. In (A), (B) and (C) the results show enzimatic activity in the absence (+) and the presence of Hcy 5 mM (▲) and Hcy 8 mM (O). ATPase, ADPase and AMPase activities were determined in the range 0.2 – 0.4 mM of ATP, ADP and AMP respectively. Plots are representative of three isolated experiments for each nucleotide.
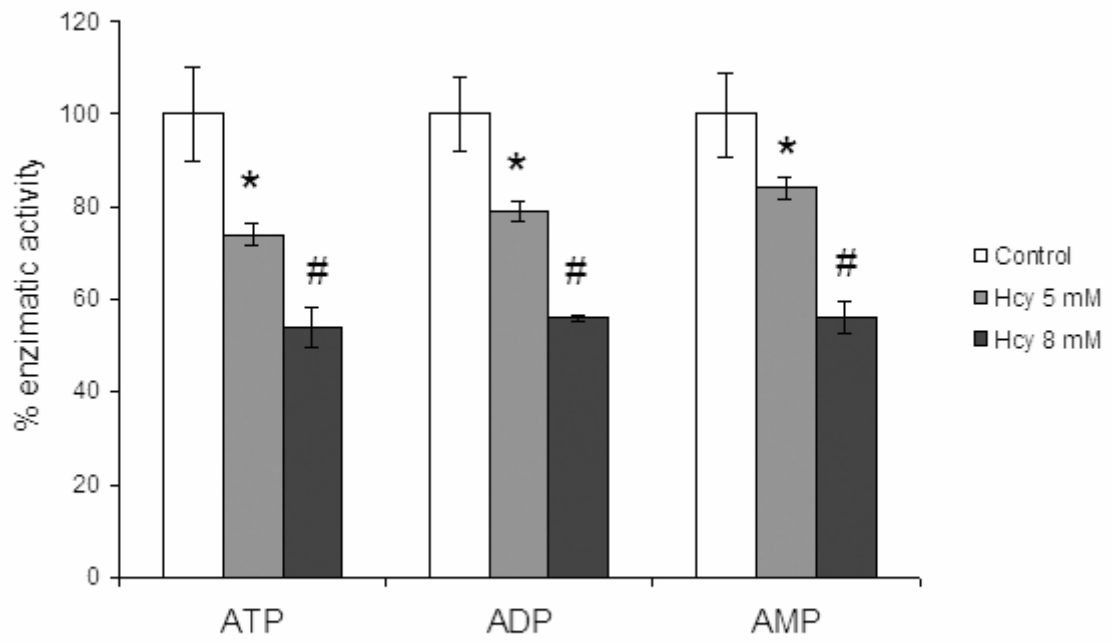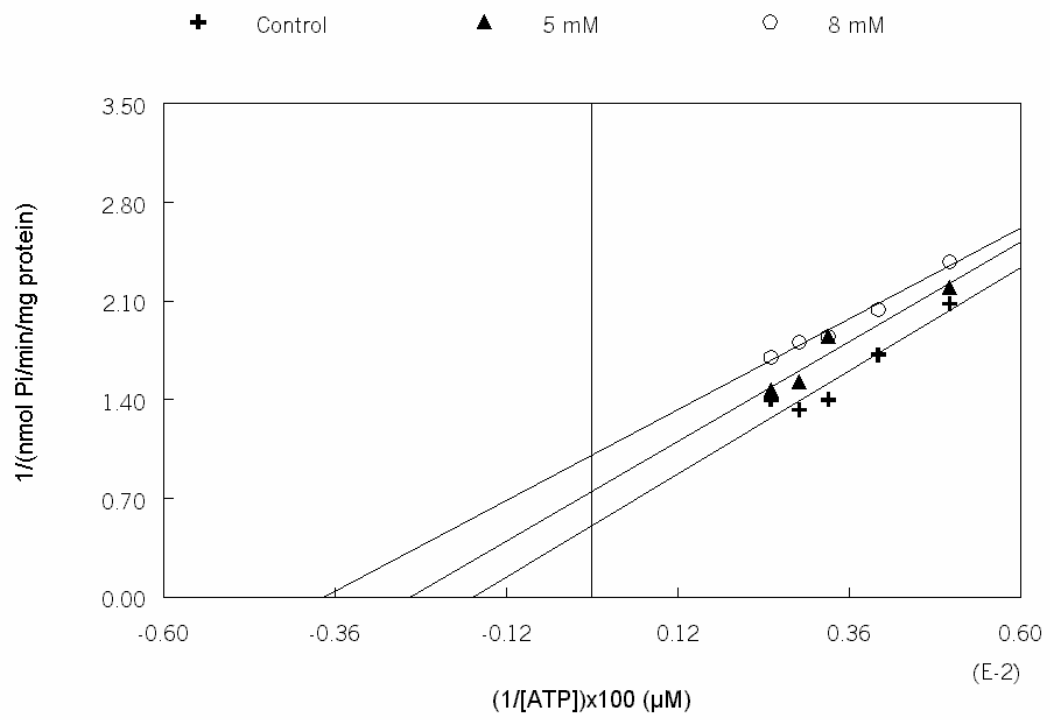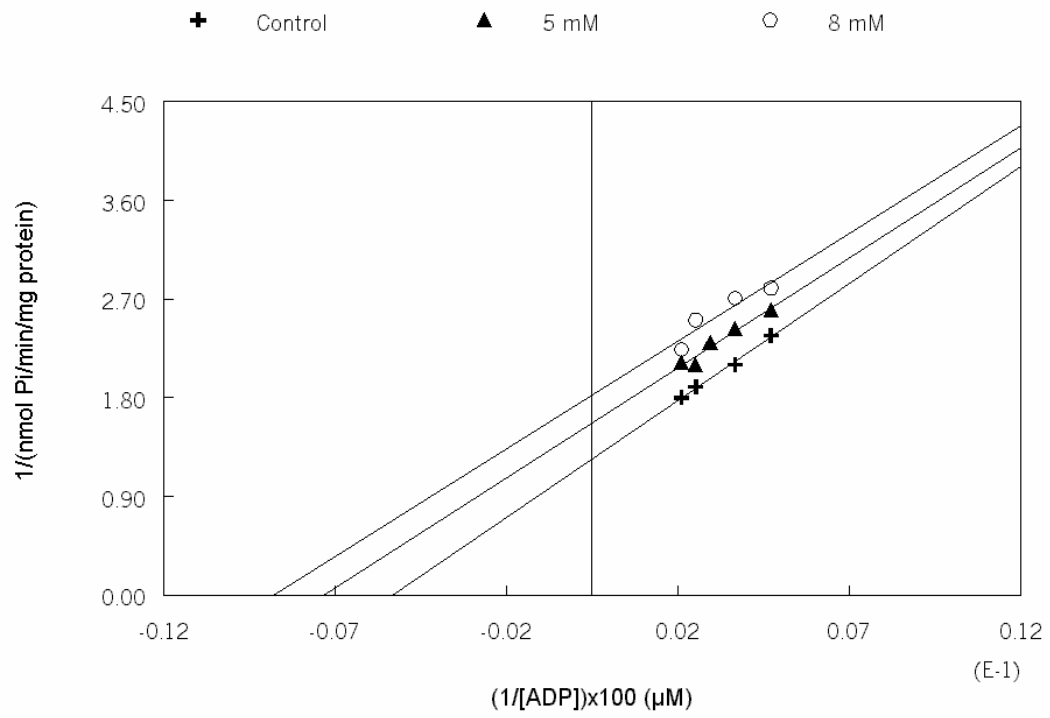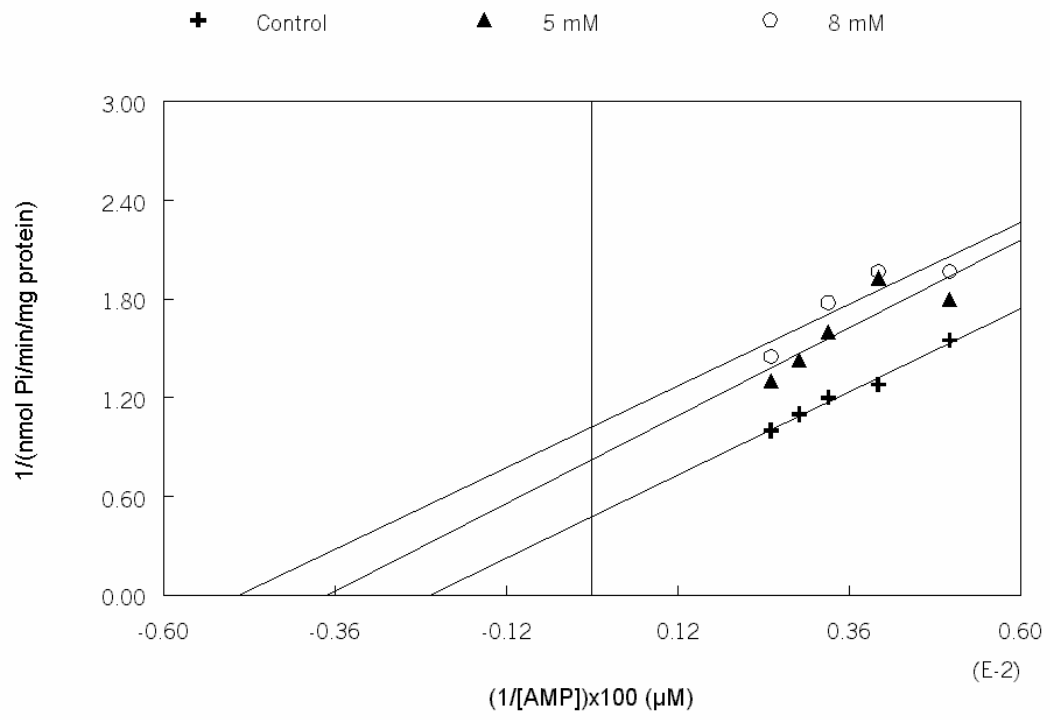
Fig. 1.

Fig. 2A

Fig. 2B

Fig. 2C

2.2 CAPÍTULO 2

Chronic Cyclosporine Treatment Increases Total Homocysteine Level and

Decreases Adenine-nucleotides Hydrolysis in Blood Serum of Rat

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Chronic Cyclosporine Treatment Increases Total Homocysteine Level and Decreases Adenine-nucleotides Hydrolysis in Blood Serum of Rat

Ana Elisa Böhmer [a], Liz Marina B. P. Brum [b], Carolina G. Souza [a], Jean P. Oses [a], Mariana Streit [a], Ricardo S. Bruch [b], João J.F. Sarkis [a], Luis V. Portela [a], Diogo O. Souza [a]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600- Anexo, CEP 90035-003, Porto Alegre, RS, Brazil.

[b] Instituto de Cardiologia do Rio Grande do Sul, FUC, Porto Alegre, RS, 90620-001 Brazil.

* Address correspondence to:

Dr. Diogo Onofre Souza

Universidade Federal do Rio Grande do Sul

Departamento de Bioquímica, ICBS

Rua Ramiro Barcellos, 2600-ANEXO

CEP 90035-003, Porto Alegre, RS, Brazil

Phone number: +55 (51) 33165557

Fax number: +55 (51) 33165540

E-mail address: diogo@ufrgs.br

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Abstract

Objective: Cyclosporine, a potent immunosuppressant agent that has been extensively used in transplanted patients, is related to a variety of side effects. Vascular disease is a major cause of morbidity and mortality among renal and cardiac transplant recipients, but the underlying mechanisms of vascular injury caused by cyclosporine are poorly understood. Here, we examined the effects of long-term cyclosporine administration on total homocysteine levels, adenine-nucleotides hydrolysis, and its putative association with vascular disease.

Methods: Male Wistar rats were divided in three groups The control group received vehicle (corn oil), and treated groups received cyclosporine 5 mg/kg or 15 mg/kg, by daily gastric gavage during 8 weeks. Concentrations of cyclosporine, fibrinogen, platelets and total homocysteine were assessed, as well as ATP, ADP and AMP hydrolysis in blood serum.

Results: Cyclosporine induced a statistically significant increase in total homocysteine, fibrinogen levels, and platelets number, whereas induced a decrease in uric acid levels and ATP, ADP and AMP hydrolysis. The inhibition of nucleotides hydrolysis correlated negatively with total homocysteine levels and positively with uric acid levels.

Conclusions: Cyclosporine might create a favorable scenario for a thrombotic state by increasing platelets, fibrinogen and serum levels of total homocysteine, which in turn affects the hydrolysis of serum adenine nucleotides, compounds known to be involved in haemostasis, thrombosis and inflammation processes.

Key words: Cyclosporine, homocysteine, nucleotide hydrolysis, thrombosis.

1.   Introduction

Cyclosporine (CsA) is a potent immunosuppressant agent used extensively in autoimmune diseases and in organ transplants to prevent graft rejection [1-3]. The use of CsA is accompanied by a variety of side effects, including microvascular thrombosis and neurotoxicity [4]. Moreover, cardiovascular disease is a major cause of morbidity and mortality among renal and cardiac transplant recipients [5,6]. However, the underlying mechanisms of vascular injury caused by CsA in transplanted patients are poorly understood, probably due to multifactorial responses caused by the presence of the allograft. There are emerging evidences showing that CsA may exert direct action on vascular endothelial cells as well as trigger an increase in serum fibrinogen and platelet aggregation [7-10].

It has been suggested that CsA affects the homocysteine (Hcy) serum concentrations [11]. This sulfur containing amino acid is widely accepted as an independent risk factor for atherosclerosis [12], peripheral vascular disease [13], brain injury [4], myocardial infarction [14] and venous thromboembolism [15]. Hcy-induced vascular damage appears to be related to increased production of free radicals and platelet adhesiveness [16,17], and also to decreased tissue and plasmatic adenosine (Ado) concentrations [18-20].

The involvement of extracellular nucleotides and nucleosides in various biological processes has gained much interest in the last years [19,21]. Extracellular blood nucleotides are released by leukocytes, endothelium cells and platelets, and provide poorly characterized, yet ubiquitous, environmental signals within the blood vessels [22,23]. Extracellular ATP has been suggested to play a role in vascular tone, cardiac function and renal epithelial transport [24], and ADP

is known to induce changes in platelet shape and aggregation. Several authors have described the important role of these nucleotides in the processes of haemostasis, thrombosis and inflammation [25-29]. The extracellular nucleoside adenosine derived from nucleotide hydrolysis and cell release has important benefic effects, such as vasodilatation and inhibition of platelet aggregation, avoiding thrombus formation and circulatory problems [24].

Extracellular nucleotides concentration can be regulated by the action of ecto- and soluble nucleotidases [30], including Ecto-ATP diphosphohydrolase (EC 3.6.1.5, NTPDase, ecto-apyrase, CD39), a member of NTPDase family, able to hydrolyze ATP, ADP and other triphospho- and diphosphonucleosides to their equivalent monophosphonucleoside [31,32]. This ecto or soluble enzyme acts together with 5'-nucleotidase (EC 3.1.3.5, CD73), which hydrolyses monophosphonucleosides, as AMP, to inorganic phosphate and adenosine [32]. Therefore, this enzymatic cascade is able to regulate nucleotide/nucleoside-mediated responses within the vascular system.

The aim of this study was to determine the effects of chronic CsA administration on total serum Hcy (tHcy) levels, adenine nucleotide hydrolysis, and its putative association with vascular disturbance.

2.   Methods

2.1.   Animals and Reagents

Male Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, Porto Alegre, RS, Brazil. Adult rats,

60 days old, weighting around 250 grams, were maintained under a standard dark-light cycle, at a room temperature of 22 + 2°C, with free access to food and water. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Rats were divided in three groups according to the treatment schedule. The control group received corn oil, and CsA-treated groups received CsA 5 mg/kg or CsA 15 mg/kg diluted in corn oil. Administrations were performed by daily gastric gavage during 8 weeks. After anesthesia with sodium thiopental (40 mg/kg), blood samples were collected by cardiac punction 24 hours after the last vehicle or CsA administration.

CsA concentrations in whole blood were determined by enzyme multiplied immunoassay test (EMIT-Green Liquid, Dade-Behring on Cobas Mira, Roche Diagnostic Systems, USA). Serum tHcy concentrations were measured using a commercial MEIA kit (Abbott, USA). Platelets were counted using automatized equipment (Penta 60, ABX, France). Fibrinogen was measured using Fibritimer-II (Dade Behring, Germany) equipment. Serum uric acid was analyzed using commercial kits manufactured by Roche (USA) in automatized equipment (Cobas Integra 400, Roche, USA). Nucleotides were purchased from Sigma Chemical Co., St Louis, MO, USA. All others reagents were of analytical grade.

2.2. *Measurement of ATP and ADP hydrolysis in rat serum*

ATP and ADP hydrolysis were evaluated using the method described by Oses et al. [33]. The reaction mixture containing 3.0 mM ADP or ATP as substrate, 1.0 - 1.5 mg serum protein and 112.5 mM Tris-HCl, pH 8.0, was

incubated at 37o C for 40 minutes in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of 10% TCA. All samples were centrifuged at 5000 g for 5 min and the supernatant was used for measuring the amount of inorganic phosphate (Pi) released through a colorimetric assay [34]. Incubation times and protein concentrations were chosen to ensure the linearity of the reaction (results not shown). In order to correct non-enzymatic hydrolysis, we performed controls by adding serum after TCA. All samples were assayed in duplicate. Enzyme activities were expressed as nmol of Pi released per minute per milligram of protein.

2.3. Measurement of AMP hydrolysis in rat serum

To evaluate the AMP hydrolysis, we used a reaction mixture containing 3.0 mM AMP in 100 mM Tris-HCl, pH 7.5, incubated with 1.0 – 1.5 mg of serum protein at 37° C in a final volume of 0.2 mL. All other procedures were the same as described above for ATP and ADP hydrolysis.

2.4. Protein determination

Protein was measured by the Coomassie Blue method [35], using bovine serum albumin as standard.

2.5. Data analysis

Data were analyzed using one-way ANOVA, followed by the Tukey's multiple range tests. Pearson's test was used to determine correlations. $P < 0.05$ was considered to represent a significant difference in the statistical analysis, which was performed using the Statistical Package for Social Sciences (SPSS) software. Data for serum concentrations are expressed as mean ± S.D., whereas nucleotide hydrolyzes data are expressed as mean ± S.E.M..

3.   Results

Twenty-four hours after the last administration, blood CsA concentration in CsA 15 mg/kg group was statistically significant different (425.7 ± 66.5 ng/mL, n=10) compared to CsA 5 mg/kg group (92.4 ± 30.1 ng/mL, $p < 0.001$, n=10). No detectable CsA level was found in control group (n=10).

Mean tHcy level increased in CsA 15 mg/kg group (8.2 ± 1.2 µmol/L, n=14) compared to CsA 5 mg/kg (5.4 ± 0.5; $p < 0.001$, n=14) and control (4.7 ± 0.7 µmol/L; $p < 0.001$, n=14) groups.

In CsA 15 mg/kg treated group there was an increase in platelet number (68.4 ± 9.3 x 10^4/mm3, n=14) in comparison to control group (58.4 ± 3.8 x 10^4/mm3, $p < 0.05$, n=14). There was no difference in platelet number between CsA 5 mg/kg (64.4 ± 5.6 x 10^4/mm3, n=14) and the other groups.

Fibrinogen levels were also increased in CsA 15 mg/kg group (596.0 ± 258.0 mg/dL, n=9) when compared to CsA 5 mg/kg (347.0 ± 47.7, mg/dL, $p < 0.01$, n=9) and control (338.0 ± 85.8, mg/dL, $p < 0.01$, n=9) groups.

3.1. ATP, ADP and AMP hydrolysis

The effects of CsA administration on ATP, ADP and AMP hydrolysis by serum of rats are shown in Fig. 1. When compared to controls, the animals submitted to CsA treatment presented statistically significant decrease in ATP hydrolysis. CsA 5 mg/kg and CsA 15 mg/kg groups showed 27% and 37% of inhibition, respectively, when compared to control (0.49 ± 0.12 nmol Pi/min/mg, $p < 0.05$, n=7). The ADP hydrolysis was also decreased in CsA 15 mg/kg (46%) and CsA 5 mg/kg group (36%), respectively, compared to control group (0.83 ± 0.18 nmol Pi/min/mg, $p < 0.05$, n=7). The results of the 5'-nucleotidase show a similar profile of inhibition observed in ATP and ADP hydrolysis: a statistically significant decrease of the 5'-nucleotidase activity in CsA 5 mg/kg (34%) and CsA 15mg/kg (34%) compared to control group (1.36 ± 0.17 nmol Pi/min/mg, $p < 0.05$, n=7).

Uric acid levels were decreased in CsA 5 mg/kg group (0.88 ± 0.10 mg/dL, $p < 0.001$, n=7) and CsA 15 mg/kg (0.85 ± 0.14 mg/dL, $p < 0.001$, n=7) when compared to controls (1.48 ± 0.38 mg/dL, n=7). There was a statistically significant correlation between uric acid levels and ATP ($r = 0.678$, $p < 0.001$), ADP ($r = 0.642$, $p < 0.002$) and AMP ($r = 0.764$, $p < 0.001$) hydrolysis.

There was a highly statistically significant inverse correlation among tHcy levels with ATP, ADP and AMP hydrolysis (Fig. 2).

4.   Discussion

The calcineurin inhibitor CsA has been the basis for most immunosuppressive protocols for more than two decades. Unfortunately, adverse effects of CsA treatment such as nephrotoxicity, neurotoxicity, hypertension, and obliterative vascular disease have become the major limiting factors in long-term

immunosuppressive therapy of transplanted patients [5,6,36,37]. Allograft vasculopathy has emerged as one of the primary causes of morbidity and mortality in long-term transplanted patients, surpassing even infection [38]. The action of CsA as an independent factor for vascular alterations and the possible mechanisms involved are difficult to delimit after transplant due to multiple confounding factors in the clinical setting. In the present study, we administered CsA in non-transplanted rats, in order to evaluate CsA effects on parameters related to vascular disturbances.

Accordingly, there are some reports of increased serum tHcy levels in transplant recipients [11,39-42], which have been proposed as a contributing agent for accelerated allograft vasculopathy [2]. In our study, CsA 15 mg/kg increased tHcy concentrations in blood serum, and such increment could be consequence of the inhibitory action of CsA on folate-dependent remethylation of Hcy to form methionine. However, further studies addressing the mechanisms involved in tHcy increase are necessary to elucidate this speculation.

It is recognized that increased serum Hcy level is an important and independent risk factor for cardiovascular diseases [15,43]. Hcy has been associated with loss of endothelial antithrombotic function and induction of a procoagulant milieu [44]. Moreover, moderate elevation in plasma Hcy levels may alter vascular morphology, stimulate inflammation and blood-clotting cascade, causing damage to endothelium and inhibiting fibrinolysis [45,46]. Platelets activation contributes to thrombotic, ischemic, and inflammatory processes [47]. Likewise, fibrinogen has a role as an inflammatory acute phase protein and strongly affects erythrocyte sedimentation rate [48] and increases platelet aggregability [49]. Thus, the increased platelets number and fibrinogen levels

observed in the present study support the postulation of an inflammatory and pro-thrombotic state associated with CsA treatment.

Chen et al. [20] demonstrated that a decrease in plasma adenosine levels might be an important event resulting in pathological cardiovascular processes. Moreover, the cardiovascular complications reported in patients with severe hyperhomocisteinemia are associated to a decrease of beneficial effects provided by adenosine [18]. Although vascular disorders can be also associated to an imbalance in the ratio of nucleotides/nucleosides in the circulation, the effects of CsA on adenine nucleotides hydrolysis has received little attention. The coordinated activities of soluble NTPDase and 5'nucleotidase are able to regulate nucleotide/nucleoside-mediated responses within the vasculature [32]. In the present study, CsA treated rats had a decrease in ATP, ADP and AMP hydrolysis activity in serum. Moreover, the observed decreased serum levels of uric acid, the catabolic end product of purines, which is significantly correlated with nucleotides hydrolysis activities, corroborated these results. In the cardiovascular system, ATP and other nucleotides can be released by cell lyses and/or cell death as well as exocytosis. Their biological effects are mainly determined by their rate of release in the extracellular medium, the activity of ecto-nucleotidases and their binding affinity to specific receptors. Inhibition of nucleotidases may prolong the effect of nucleotides [50, 51]. Extracellularly, ATP has important vascular actions [52]; in elevated concentrations it induces vasoconstriction and promotes its own stimulated release from endothelial cells. Additionally, ADP is a potent platelet-recruiting factor inducing platelet aggregation via binding of platelet P2Y12 receptors [53]. The inhibition observed in serum ATP and ADP hydrolysis by CsA treatment could enhance the ATP-induced vasoconstriction [54] and the platelet

aggregation mediated by ADP, the most potent platelet agonist [55]. Moreover, inhibition of AMP hydrolysis may contribute to a decrease in adenosine levels, a cardiovascular protective molecule that inhibits platelet aggregation, dilates coronary and cerebral arteries, increases blood flow and decreases proliferation or growth of vascular smooth muscle cells [56,57]. The strong inverse correlation between tHcy levels and adenine nucleotides hydrolysis suggests that the inhibition of ecto-nucleotidase activities could be Hcy dependent. This hypothesis is supported by recent *in vitro* studies of our laboratory, which demonstrated that Hcy inhibits, in an uncompetitive way, the ATP, ADP and AMP hydrolysis by blood serum of rats (data not shown).

In summary, our results demonstrate that CsA long-term treatment induces vascular disturbances. CsA might create a favorable scenario for a pro-thrombotic state, increasing platelets and fibrinogen levels. Additionally, it increases tHcy serum concentrations and inhibits serum ATP, ADP and AMP hydrolysis, probably decreasing serum adenosine levels and therefore its beneficial effects on the cardiovascular system. Low levels of uric acid during CsA treatment point that this inhibition has *in vivo* relevance. Altogether, these alterations could be implicated in the vascular complications reported in patients under CsA therapy.

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Figure Legends

Fig. 1. Effect of cyclosporine administration on ATP, ADP and AMP hydrolysis by rat blood serum. Values are expressed as mean ± S.E.M. for seven independent experiments. Results are expressed as specific activity (nmol of Pi/min/mg). Control group values for ATP, ADP and AMP hydrolysis were 0.49 ± 0.12, 0.83 ± 0.18 and 1.36 ± 0.17, respectively. Absolute values for CsA 5 mg/kg groups were: 0.36 ± 0.16, 0.53 ± 0.20 and 0.90 ± 0.20; and for CsA 15 mg/kg group were: 0.31 ± 0.06, 0.45 ± 0.09 and 0.90 ± 0.18 for ATP, ADP and AMP hydrolysis respectively. * indicates significant difference from control group ($p < 0.05$). Statistical analysis was performed using one-way ANOVA, followed by the Tukey's multiple range test.

Fig. 2. Correlations among total homocysteine serum levels and ATP (A) n=20, ADP (B) n=20, and AMP (C) n=18 hydrolysis by blood serum of rats treated with cyclosporine. There were statistically significant negative correlations between homocysteine levels and nucleotides hydrolysis, by Pearson's correlation test.
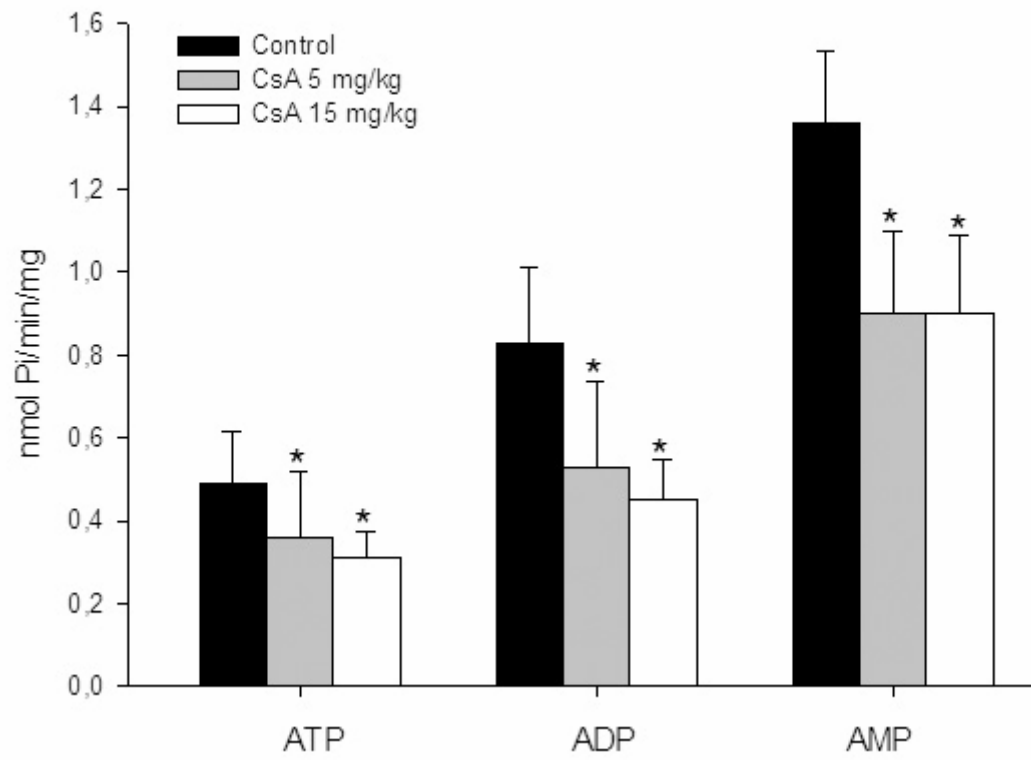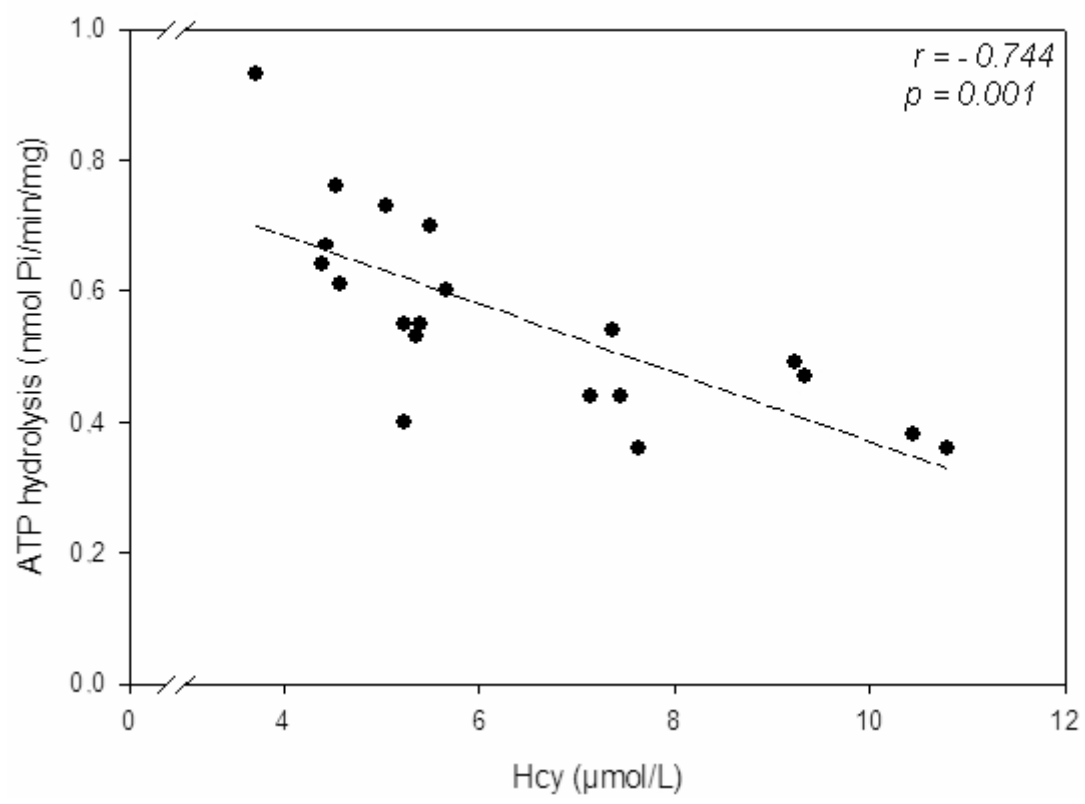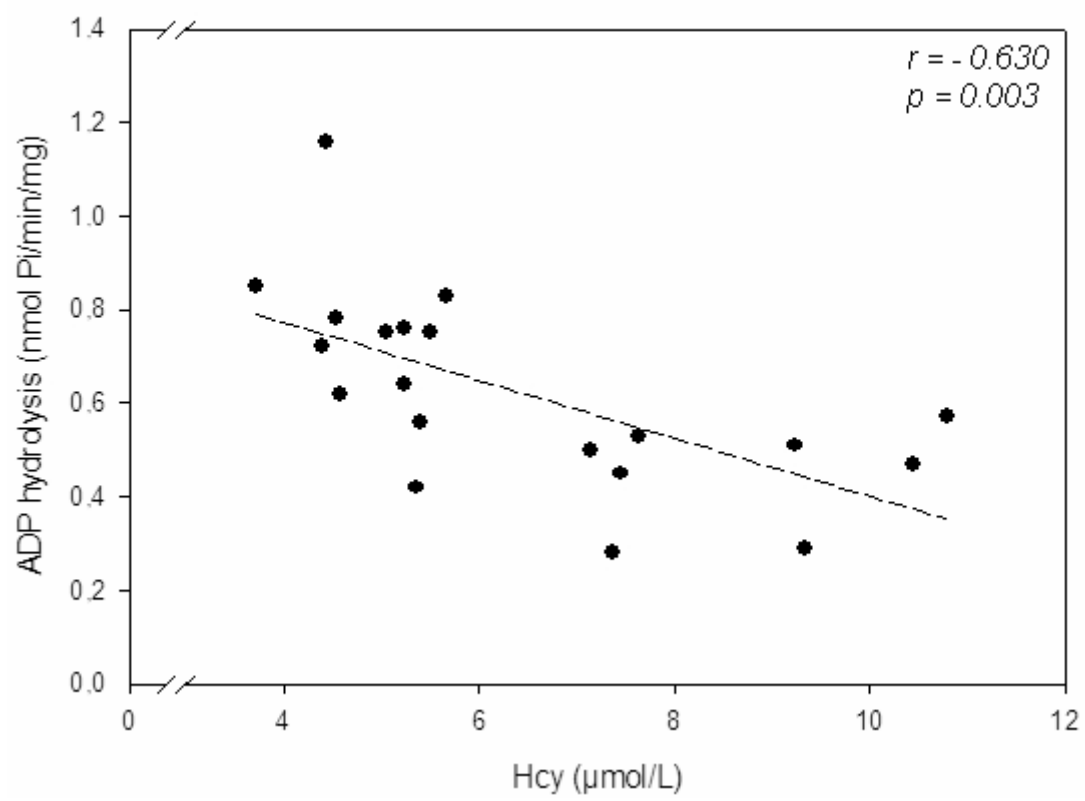
Fig. 1.

Fig. 2A

r = - 0.744
p = 0.001

Fig. 2B

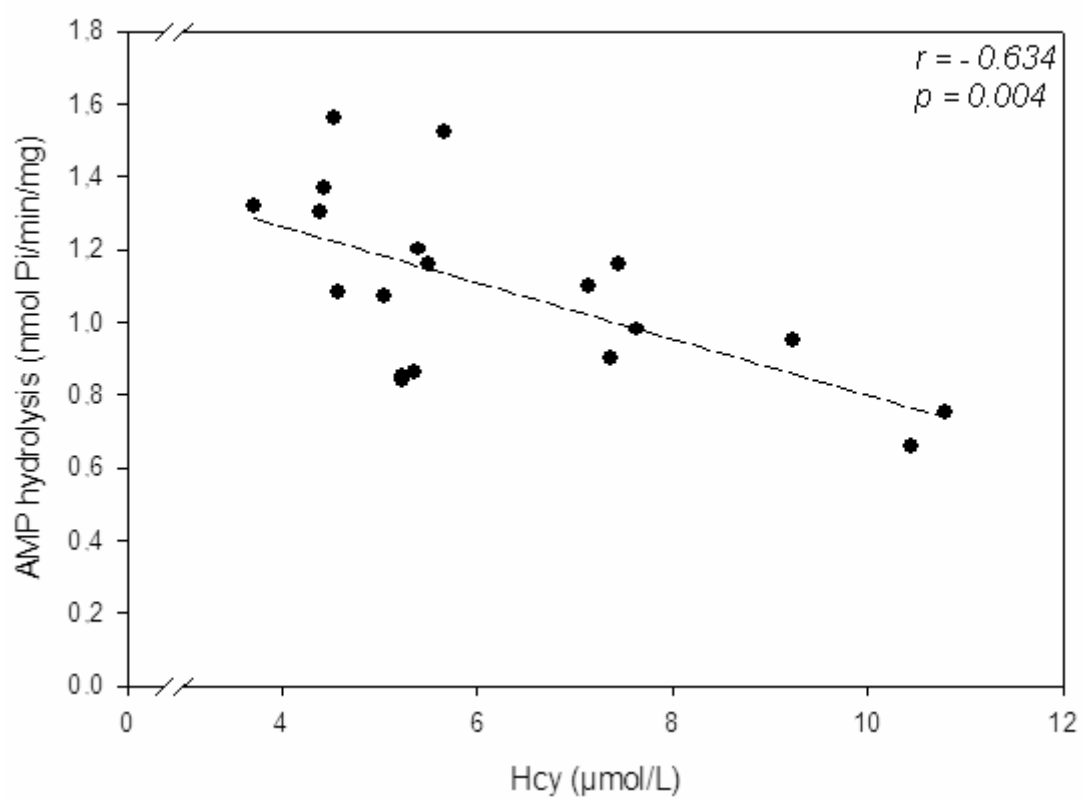Fig. 2C

4. DISCUSSÃO

Alguns pesquisadores têm proposto o aumento das concentrações de tHcy como um fator de risco independente para doenças cardiovasculares (BOUSHEY et al., 1995; MALINOW, 1996; MAYER, JACOBSEN & ROBINSON, 1996; MINER, EVROVSKI & COLE, 1997; UPCHURCH et al, 1997; JACOBSEN, 1998; DEN HEIJER et al., 1998; STANGER et al., 2004). O sistema cardiovascular é particularmente sensível aos níveis de tHcy circulante (CHEN et al., 1999), o que significa que o aumento de tHcy pode acarretar em alterações da morfologia vascular, inflamação vascular, danos ao endotélio e ativação da cascata de coagulação e conseqüente indução de um estado aterotrombótico (MALINOW, 1996; UPCHURCH et al, 1997; DEN HEIJER, 1998; STANGER et al., 2004).

Os mecanismos pelos quais a Hcy induz disfunções cardiovasculares não estão bem estabelecidos, no entanto, há estudos que sugerem o aumento do estresse oxidativo e produção de citocinas pró-inflamatórias causados pela Hhcy como fatores que contribuem para essas alterações (WELCH, UPCHURCH & LOSCALZO, 1997; HANKEY & EIKELBOOM, 1999; PODDAR, 2001; DALAL, 2003; FARACI, 2003; FARACI & LENTZ, 2004; SU, 2005; TYAGI, 2005). Recentemente, tem sido descrito que a Hcy altera as concentrações plasmáticas e teciduais de adenosina, um nucleosídeo derivado de nucleotídeos da adenina (AGTERESCH, 1999 ; DEUSSEN, 1999; Chen, Li & Zou, 2002; DEUSEN, 2003; RIKSEN et al., 2003; SELLEY , 2004; DEUSSEN , 2005; RIKSEN et al., 2005). Portanto, as disfunções vasculares podem estar relacionadas ao desequilíbrio das concentrações de nucleotídeos/nucleosídeo na circulação.

As enzimas nucleotidases localizadas nas membranas plasmáticas de diversas células ou solúveis no meio intersticial representam um importante mecanismo de controle dos níveis de ATP, ADP e AMP na circulação, sendo a sua regulação importante para a homeostasia, trombo-regulação e outros aspectos da sinalização purinérgica (ZIMMERMANN, 2001). Neste sentido, a interação da Hcy com o metabolismo de nucleotídeos e alterações vasculares tem recebido muita atenção por parte do nosso grupo de pesquisa.

Em nossos estudos, investigamos o efeito *in vitro* da Hcy sobre a hidrólise dos nucleotídeos extracelulares ATP, ADP e AMP em soro de ratos. Os resultados demonstram que a Hcy inibe de forma significativa a hidrólise dos três nucleotídeos e de forma dependente da concentração de Hcy. Investigamos também o tipo de inibição causada pela Hcy sobre as nucleotidases envolvidas no processo de degradação desses nucleotídeos. As análises cinéticas demonstraram que a Hcy inibe a hidrólise de forma acompetitiva, ou seja, liga-se somente ao complexo enzima + substrato.

Este tipo de inibição freqüentemente ocorre com enzimas que se ligam ao substrato de maneira ordenada, ou seja, se ligam primeiro ao ATP depois ao ADP. Este comportamento sugere a ação de uma NTPDase tipo ATP-difosfoidrolase.

A atividade NTPDásica e 5'-nucleotidásica tem sido sugerida como um mecanismo importante para o completo metabolismo dos nucleotídeos extracelulares (ZIMMERMANN et al., 2001) e a inibição das atividades nucleotidásicas acarreta o prolongamento dos efeitos vasculares causados pelos nucleotídeos ATP, ADP e AMP em seus respectivos receptores (IMAI et al., 1999; GENDRON et al., 2002). Por exemplo, altas concentrações de ATP podem

induzir vasoconstrição da parede vascular e ao mesmo tempo, estimular a liberação de mais ATP pelas células endoteliais (BURNSTOCK, 1990; KONISHI, C.; NAITO, Y.; OHARA, 1999). O ADP, importante fator de ativação plaquetária, em concentrações mais elevadas na circulação, estimula a agregação plaquetária via receptores $P2Y_{12}$ (PURI & COLMAN, 1997; GACHET, 2001). Assim, a inibição da hidrólise destes nucleotídeos propicia a formação de um estado pró-trombótico e poderia estar contribuindo para o desenvolvimento de doenças vasculares observadas em pacientes com Hhcy.

Além disso, a inibição causada pela Hcy sobre a hidrólise de AMP também diminuiu a formação de adenosina, uma molécula com importantes funções protetoras para o sistema cardiovascular, como vasodilatação, inibição da agregação plaquetária e inflamação (BERNE, 1963; RALEVIC & BURNSTOCK, 2003). A inibição causada pela Hcy sobre a hidrólise dos nucleotídeos ATP, ADP e AMP em soro representa uma diminuição da produção de adenosina. Assim, além da atividade reversa da S-adenosilhomocisteína hidrolase (RIKSEN et al., 2003; RIKSEN et al., 2005), a inibição da atividade das enzimas NTPDase e 5'-nucleotidase em soro contribui para a compreensão dos níveis diminuídos de adenosina circulante em pacientes com Hhcy e ressalta um importante mecanismo de ação da Hcy na etiopatogenia das doenças cardiovasculares.

Além das complicações cardiovasculares já relatadas em pacientes que fazem uso da terapia imunossupressora com CsA (HOSENPUD, SHIPLEY & WAGNER, 1992; WEIS & VON SCHEIDT, 1997), procuramos também elucidar possíveis mecanismos de ação pelo qual a CsA exerce efeitos deletérios cardiovasculares. Para tanto, em soro de ratos tratados com CsA analisamos parâmetros relacionados à coagulação e homeostasia vascular como fibrinogênio

e plaquetas, níveis circulantes de tHcy e a hidrólise dos nucleotídeos ATP, ADP e AMP.

Observamos que o tratamento imunossupressor crônico com CsA alterou parâmetros de coagulação, como plaquetas e fibrinogênio. Alguns trabalhos demonstram que além do aumento do número de plaquetas a CsA também aumenta a agregação plaquetária (MALYSZKO et al., 1996; REIS et al., 1999). O tratamento com CsA também aumentou os níveis de fibrinogênio, uma proteína de fase aguda que está relacionada com a formação de placas ateroscleróticas (MAURIELLO et al., 2000) e trombos (SABETI et al., 2005). Os níveis aumentados de fibrinogênio alteram a viscosidade sangüínea, a sedimentação de eritrócitos (VAN LENTE, 2000) e a agregação plaquetária (LEFKOVITS, PLOW & TOPOL, 1995). Desta forma, o aumento do número de plaquetas e dos níveis de fibrinogênio ressalta um estado pró-inflamatório e pró-trombótico induzido pela CsA.

Como já relatado em outros estudos (AMBROSI et al., 1994; ARNADOTTIR et al., 1996; ARNADOTTIR et al., 1998; COLE et al., 1998; GUPTA et al., 1998; HERRERO et al., 2000) a administração de CsA aumentou os níveis de tHcy em ratos tratados. No entanto, os mecanismos envolvidos neste aumento de tHcy não estão bem esclarecidos. É possível que a CsA exerça uma ação inibitória no processo de remetilação da Hcy até metionina porém, mais estudos são necessários para compreender estes mecanismos.

Em relação à hidrólise dos nucleotídeos de adenina, o tratamento com CsA inibiu a hidrólise de ATP, ADP e AMP em soro e também diminuiu os níveis de ácido úrico circulantes (produto final da degradação de purinas). Além disso, a inibição das atividades NTPDásica e 5'-nucleotidásica se correlacionaram

negativamente com o aumento de tHcy, sugerindo que a diminuição da hidrólise de ATP, ADP e AMP em soro de ratos tratados com CsA seja dependente do aumento das concentrações de Hcy.

Os resultados encontrados em ratos tratados com CsA estão de acordo com aqueles relatados para a hidrólise de nucleotídeos *in vitro*, em que a Hcy inibiu a hidrólise de ATP, ADP e AMP de forma acompetitiva e dependente da concentração de Hcy.

Desta forma, podemos concluir que o tratamento crônico com CsA altera parâmetros importantes da circulação vascular. O aumento dos níveis de fibrinogênio e plaquetas associados ao aumento de tHcy e o desequilíbrio dos níveis nucleotídeos/nucleosídeo circulantes aumenta o risco de eventos aterotrombóticos. Além disso, estes resultados contribuem para a compreensão dos mecanismos envolvidos nas complicações vasculares relatadas em pacientes que fazem uso da CsA.

Até o presente momento não há nenhum dado na literatura com respeito a alterações na atividade de nucleotidases solúveis em modelos de imunossupressão com CsA. A relação entre CsA, os níveis de tHcy circulante e as enzimas que controlam os níveis de nucleotídeos em soro de ratos merece atenção, uma vez que podem estar envolvidos nas disfunções cardiovasculares em pacientes que fazem uso da terapia imunossupresiva.

Portanto, as evidências obtidas neste trabalho podem ser relevantes para futuros trabalhos que tenham por objetivo investigar doenças cardiovasculares em pacientes com Hhcy e/ou que fazem uso da terapia imunossupressora com CsA. Dosagens de tHcy, atividades nucleotidásicas, ácido úrico e adenosina

circulante podem ser de grande utilidade para o monitoramento dos efeitos adversos do tratamento com CsA.

5. CONCLUSÕES

- A Hcy alterou *in vitro*, de forma dependente da concentração, a hidrólise dos nucleotídeos ATP, ADP e AMP em soro de ratos.

- A Hcy inibiu de forma acompetitiva a atividade da NTPDase e 5'-nucleotidase envolvidas na hidrólise de ATP, ADP e AMP em soro de ratos.

- Em pacientes com Hhcy, a inibição da atividade de enzimas NTPDase e 5'-nucleotidase parece estar envolvida na etiopatogenia dos problemas cardiovasculares comumente descritos.

- O tratamento imunossupressor com CsA aumentou o número de plaquetas e os níveis de fibrinogênio no sangue dos ratos. Parâmetros que estão envolvidos em processos aterotrombóticos.

- A CsA aumentou os níveis de tHcy em ratos tratados.

- A tratamento com CsA inibiu a atividade de hidrólise dos nucleotídeos de adenina em soro de ratos tratados e diminuiu os níveis circulantes de ácido úrico, o produto final da degradação dos nucleotídeos.

- A inibição da hidrólise dos nucleotídeos de adenina observada em ratos tratados com CsA se correlacionou negativamente com o aumento dos níveis de tHcy.

- O aumento dos níveis de tHcy e a diminuição da hidrólise de nucleotídeos parece representar um importante mecanismo de ação pelo qual a CsA contribui para o desenvolvimento das vasculopatias em pacientes imunossuprimidos.

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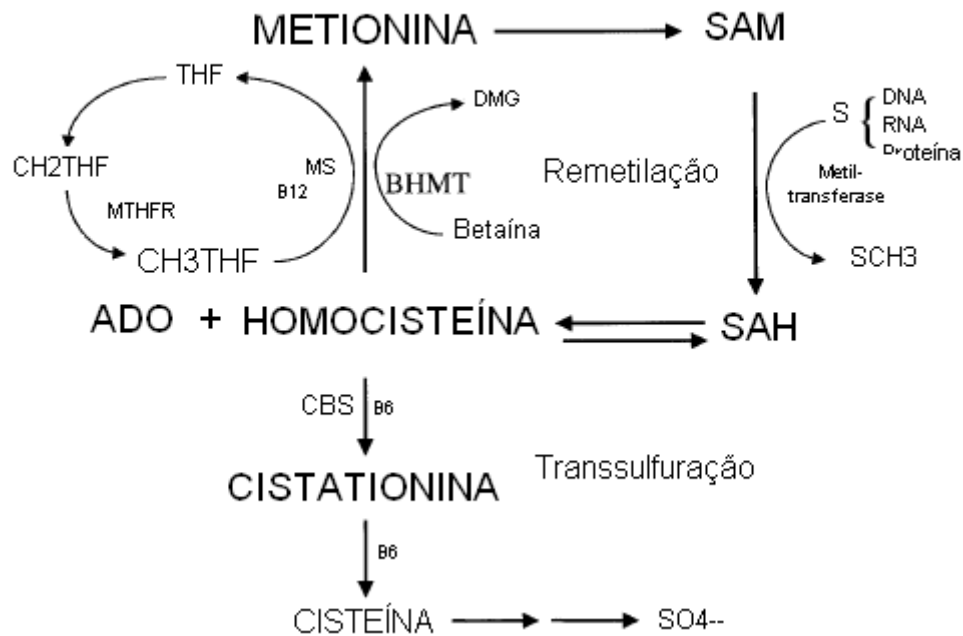
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ANEXOS

Anexo 1.

Adaptado de Jacobsen, DW. Clinical Chemistry, 1998;44(8):1833-1843.

Metabolismo da homocisteína.

Remetilação: a homocisteína é remetilada até metionina pela ação do metilenotetrahidrofolato redutase (MTHFR) e metionina sintase (MS), dependente de vitamina B12. A homocisteína também pode ser remetilada até metionina pela ação da betaína-homocisteína-metiltransferase (BHMT) no fígado e rins. A metionina é convertida em S-adenosilmetionina (SAM), que serve como substrato doador de metila para metiltransferases. Outro produto desta reação é a S-adenosilhomocisteína (SAH), que ao ser hidrolisada pela SAH-hidrolase produz homocisteína e adenosina.Transsulfuração: a primeira enzima deste processo é a cistationina beta-sintase (CBS), dependente de vitamina B6. Cistationina é convertida a cisteína, que por sua vez é catabolizada até sulfato inorgânico e excretada na urina.
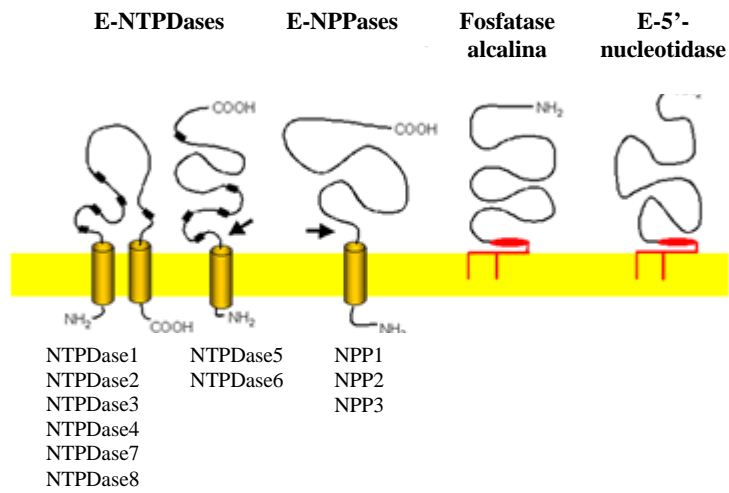
Anexo 2.
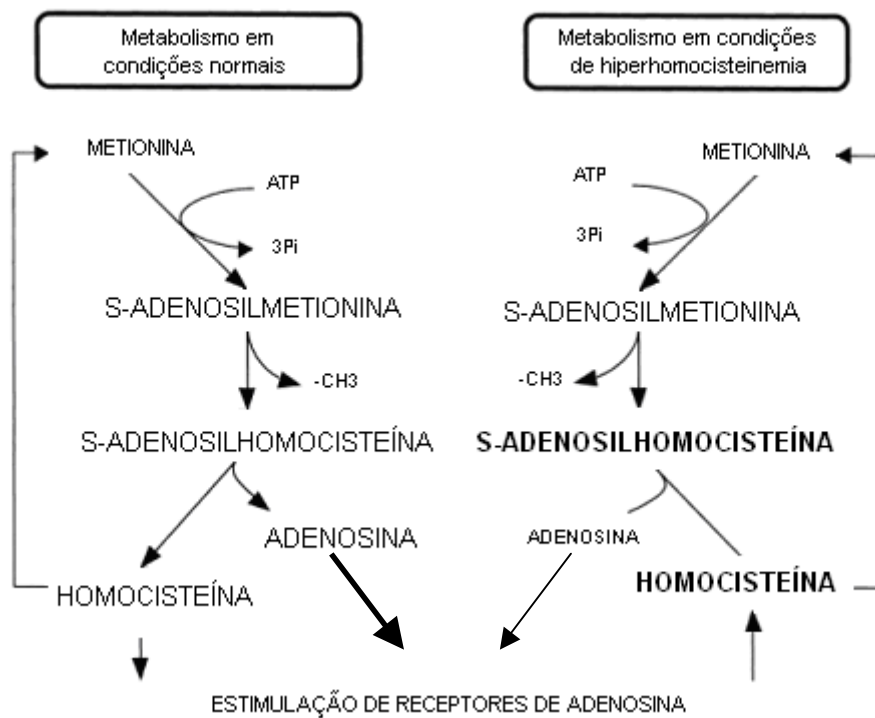
Adaptado de Riksen et al. Cardiovascular Research, 2003;59:271-276

Em condições normais, a adenosina é formada a partir da hidrólise da S-adenosilhomocisteína (SAH) (lado esquerdo do diagrama). A concentração extracelular contribui para a homeostasia do sistema vascular, através da estimulação de receptores específicos de adenosina. Em situações de altas concentrações de homocisteína, a reação catalisada pela SAH-hidrolase ocorrerá no sentido inverso (lado direito do diagrama).

Anexo 3.

E-NTPDases	E-NPPases	Fosfatase alcalina	E-5'-nucleotidase

NTPDase1   NTPDase5   NPP1
NTPDase2   NTPDase6   NPP2
NTPDase3   NPP3
NTPDase4
NTPDase7
NTPDase8

Adaptado de www.biozentrum.de/prof/zimmermann - Prof. Dr. Herbert Zimmermann.

Desenho esquemático demonstrando a estrutura espacial e topografia de membrana das Ecto-NTPDases, Ecto-NPPases, fosfatases alcalina e Ecto-5'-nucleotidases.