Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

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Alterações cerebrais, cardíacas e vasculares em ratos adultos submetidos ao modelo químico experimental de hiper-homocisteinemia leve

Emilene Barros da Silva Scherer

Orientadora: Profa Dra Angela Terezinha de Souza Wyse

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Dedico essa tese às pessoas mais importantes

da minha vida,

Cleny, minha mãe, pelo apoio constante,

Lucácio, meu marido, pelo companheirismo e amor e

Arthur, meu filho, por dar sentido a minha vida.

Viver como um rio

Viver como um rio
que vence os obstáculos
e sempre deságua no mar.
Ser persistente como um rio
que seca na estiagem,
e renasce, gota a gota,
nas chuvas do outono.
Ser gentil como um rio
que purifica os náufragos
acolhidos em suas águas.
Ser humilde como o rio
que esconde nas águas mansas
a vida efervescente.
Ser impetuoso como o rio
que rompe suas margens
arrastando a terra morta
para voltar tranquilo ao leito
depois de semear a vida.
Ser generoso como o rio
que alimenta o homem faminto
com o fruto de suas entranhas.
Viver a vida como um rio
que parece desfazer-se no mar
mas se renova a cada dia,
ao mesmo tempo frágil e eterno.

Clovis Wannmacher

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SUMÁRIO

RESUMO

A hiper-homocisteinemia leve é um fator de risco para doenças cerebrais e cardiovasculares embora os mecanismos subjacentes a essas alterações não estejam completamente elucidados. O objetivo principal desta tese de doutorado foi desenvolver um modelo experimental crônico quimicamente induzido de hiper-homocisteinemia leve em ratos adultos. Usando esse modelo, investigamos alguns parâmetros bioquímicos no sistema nervoso central e periférico, a fim de melhor compreender os mecanismos envolvidos na toxicidade mediada pela homocisteína. A hiper-homocisteinemia leve foi induzida em ratos Wistar através da administração subcutânea de homocisteína (0,03 µmol/g de peso corporal), duas vezes ao dia, do 30° a 60° dia de vida. Ratos controle receberam o mesmo volume de solução salina. Resultados demonstraram que animais submetidos a esse modelo apresentaram níveis plasmáticos de homocisteína de aproximadamente 30 µM. Em relação aos parâmetros bioquímicos, a hiper-homocisteinemia leve experimental induziu estresse oxidativo em plasma, córtex cerebral, coração e aorta, bem como diminuiu a atividade e o imunoconteúdo das subunidades catalíticas α_1 e α_2 da Na^+,K^+-ATPase em córtex cerebral e hipocampo de ratos adultos. Por outro lado, não foram observadas alterações nos níveis de mRNA transcrito da Na^+,K^+-ATPase em nenhuma estrutura cerebral estudada. A hidrólise de ATP, ADP e AMP foi significativamente diminuída em linfócitos e sinaptossomas obtidos de córtex cerebral, mas não foi alterada em soro de ratos adultos. A transcrição da ecto-5'-nucleotidase foi significativamente reduzida, mas a expressão das E-NTPDases não foi alterada em linfonodos mesentéricos de ratos submetidos à hiper-homocisteinemia leve experimental. Foi observado um aumento nos níveis de mRNA transcrito da E-NTPDase1 e ecto-5'-nucleotidase em córtex cerebral de ratos. Os níveis de ATP foram aumentados, enquanto a adenosina foi diminuída no líquor de ratos tratados com homocisteína. Em relação aos parâmetros inflamatórios, foi observado um aumento nos níveis de TNF-α, IL-1β, IL-6 e a quimiocina MCP-1 no hipocampo, enquanto os níveis de IL-1β e a IL-6 foram aumentados em córtex cerebral. Os ratos submetidos à hiper-homocisteinemia leve apresentaram um aumento na atividade da acetilcolinesterase em ambas as estruturas cerebrais testadas; o

imunoconteúdo dessa enzima foi significativamente aumentado no córtex cerebral e diminuído no hipocampo, enquanto os níveis de mRNA transcrito da acetilcolinesterase não foram alterados. Perifericamente, a homocisteína aumentou os níveis de TNF-α, IL-6 e MCP-1 em coração e os níveis de IL-6 no soro, mas não alterou a atividade da enzima butirilcolinesterase. Em conjunto, os resultados deste trabalho mostram que a homocisteína promove um estado oxidativo e inflamatório central e periférico além de prejudicar a funcionalidade de enzimas cerebrais importantes. Considerando que a hiper-homocisteinemia leve é prevalente na população e tem sido considerada um fator de risco para o desenvolvimento de doenças cerebrais e cardíacas, o presente modelo experimental pode ser útil na investigação da patofisiologia de doenças causadas pela homocisteína e estratégias de prevenção.

ABSTRACT

Mild hyperhomocysteinemia is a risk factor for cerebral and cardiovascular diseases although the mechanisms underlying these alterations are not fully understood. The main objective of this doctoral thesis was to develop a chronic chemically-induced model of mild hyperhomocysteinemia in adult rats. By using this model, we investigated some biochemical parameters in the central nervous system and peripheral, in order to better understand the mechanisms involved in homocysteine-mediated toxicity. The mild hyperhomocysteinemia was induced in rats by subcutaneous administration of homocysteine (0.03 µmol/g body weight) twice a day to the 30th to 60 day of life. Control rats received the same volume of saline. Results showed that animals subjected to this model had plasma homocysteine levels of approximately 30 µM. Results demonstrated that the experimental mild hyperhomocysteinemia elicited oxidative stress in plasma, cerebral cortex, heart and aorta and decreased the activity and the immunocontent of the α_1 and α_2 subunits of the Na^+,K^+-ATPase in cerebral cortex and hippocampus of adult rats. On the other hand, no change in levels of Na^+,K^+-ATPase mRNA transcripts in such cerebral structures. ATP, ADP and AMP hydrolysis were significantly decreased in lymphocytes and in the synaptosomal fraction of cerebral cortex, without any alteration in serum of adult rats. The transcript of the ecto-5'-nucleotidase was significantly reduced, but the expression of E-NTPDases were not altered in mesenteric lymph nodes of hyperhomocysteinemic rats. I have been observed an increase in E-NTPDase1 and ecto-5′-nucleotidase transcripts in rat cerebral cortex. ATP levels were increased, while adenosine decreased in cerebrospinal fluid of Hcy-treated rats. In relation to inflammatory parameters, was observed an increase in TNF-α, IL-1β, IL-6 and the chemokine MCP-1 in the hippocampus, while IL-1β and IL-6 levels were enhanced in cerebral cortex. Rats subjected to mild hyperhomocysteinemia presented an increase in acetylcholinesterase activity in both cerebral structures tested; the immunocontent of this enzyme was significantly increased in the cerebral cortex and decreased in the hippocampus while levels of acetylcholinesterase mRNA transcripts were not altered.

Peripherally, homocysteine increased TNF-α, IL-6 and MCP-1 levels in the heart and IL-6 levels in serum, but not altered the butyrylcholinesterase activity. Together, the results of this work show that homocysteine promotes central and peripheral oxidative and inflammatory state besides affecting the functionality of important brain enzymes. Whereas mild hyperhomocysteinemia is prevalent in the population and has been considered a risk factor for the development of brain and heart diseases, this experimental model may be useful for the investigation of patophysiology of diseases caused by homocysteine and prevention strategies.

LISTA DE ABREVIATURAS

ACh: acetilcolina
Ado: adenosina
AChE: acetilcolinesterase
ADP: difosfato de adenosina
AMP: monofosfato de adenosina
ATP: trifosfato de adenosina
BHMT: betaína homocisteína metiltransferase
BuChE: butirilcolinesterase
CAT: catalase
CBS: cistationina β-sintase
CGL: cistationina γ-liase
E-NTPDases: ecto-nucleosídeo trifosfato difosfoidrolase
E-NPPs: ecto-nucleotídeo pirofosfatase/fosfodiesterase
ERN: espécies reativas de nitrogênio
ERO: espécies reativas de oxigênio
GSH: glutationa (forma reduzida)
GPI: glicosil fosfatidil inositol
GPx: glutationa peroxidase
Hcy: homocisteína
IL-1β: interleucina1-β
IL-6: interleucina 6
LPS: lipopolissacarídeo
MAT: metionina adenosiltransferase
MCP-1: proteína quimiotática de monócito do tipo 1
Met: metionina
MS: metionina sintase
MTHFR: metileno tetrahidrofolato redutase
5-MeTHF: 5-metiltetraidrofolato
NMDA: N-metil - D- aspartato
NOS: óxido nítrico sintase
PLP: piridoxal fosfato

SAM: S-adenosilmetionina

SAH: S-adenosil homocisteína

SAHH: S-adenosil homocisteína hidrolase

SNC: sistema nervoso central

SOD: superóxido dismutase

TBARS: substâncias reativas ao ácido tiobarbitúrico

TNF-α: fator de necrose tumoral alfa

TRAP: potencial antioxidante total

VCAM-1: molécula de adesão vascular

LISTA DE FIGURAS

I. INTRODUÇÃO

1. Homocisteína

A homocisteína (Hcy) é um aminoácido sulfurado identificado pela primeira vez em 1932 por De Vigneaud que atua como um intermediário chave no metabolismo da metionina (Met). Esse aminoácido é sintetizado como um subproduto de reações de transferência de grupamento metil, as quais são importantes para a síntese de DNA, proteínas metiladas, neurotransmissores e fosfolipídios. Como ilustrado na figura 1, a Met, proveniente da dieta ou da degradação de proteínas endógenas, recebe um grupo adenosil do trifosfato de adenosina (ATP), sendo convertida em *S*-adenosilmetionina (SAM) em uma reação catalisada pela enzima metionina adenosiltransferase (MAT). A SAM é convertida por diversas metiltransferases a *S*-adenosil homocisteína (SAH) que é hidrolisada em adenosina (Ado) e Hcy através da enzima S-adenosil homocisteína hidrolase (SAHH) (Selhub, 1999; Williams and Schalinske, 2010).

Após sua síntese, a Hcy pode ser metabolizada pela via da remetilação à Met através de duas rotas: a primeira catalisada pela enzima metionina sintase (MS), que utiliza a cianocobalamina (vitamina B_{12}) como cofator para catalisar a transferência de um grupo metil proveniente do N^5-metiltetraidrofolato para a Hcy; ou da betaína, em uma reação catalisada pela betaína homocisteína metiltransferase (BHMT). A Hcy também pode ser irreversivelmente metabolizada pela via de transulfuração através da condensação com a serina produzindo cistationina, em uma reação catalisada pela enzima cistationina β-sintase (CBS), dependente de vitamina B_6 (piridoxal fosfato). No passo seguinte, a cistationina é clivada à cisteína através da reação catalisada pela cistationina γ-liase (CGL). A via de remetilação da Hcy é amplamente distribuída no organismo enquanto que a transulfuração apresenta

distribuição limitada a alguns órgãos como fígado, rins, pâncreas e intestino delgado (Brosnan et al., 2004; Finkelstein, 2007).

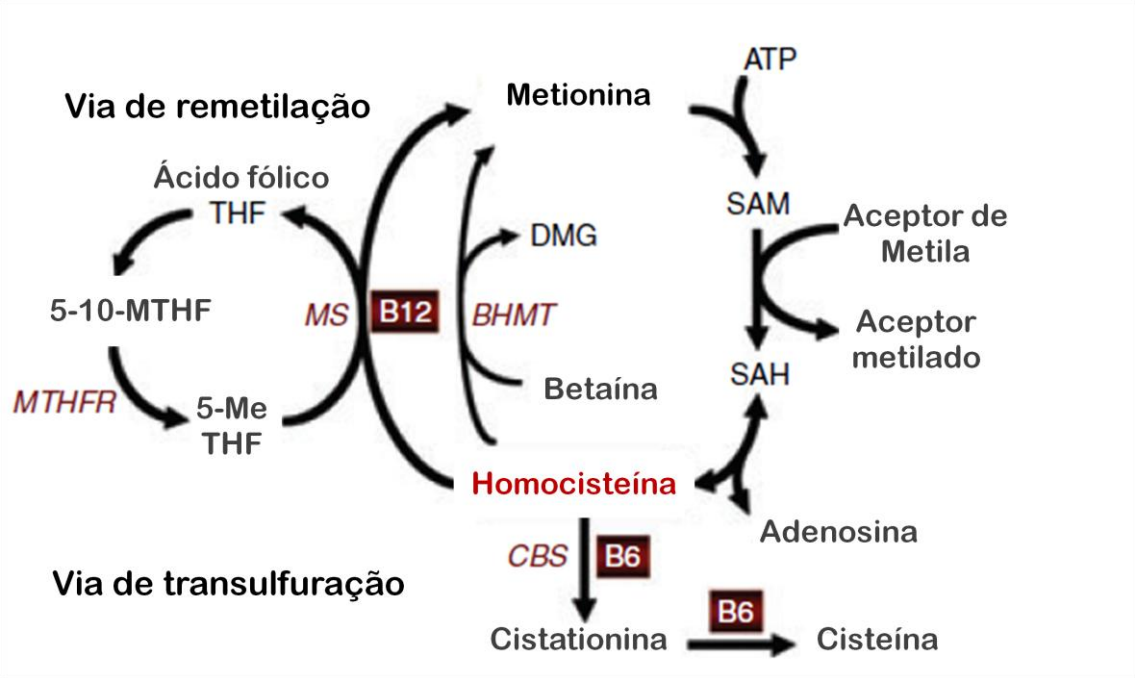

Figura 1: Metabolismo da homocisteína. SAM: S-adenosilmetionina; SAH: S-adenosil homocisteína; CBS: cistationina β-sintase; MS: metionina sintase; BHMT: betaína homocisteína metiltransferase; MTHFR: metileno tetrahidrofolato redutase; DMG: dimetil glicina THF: tetrahidrofolato; 5-10-MTHF: 5,10-metilenotetrahidrofolato; 5-Me-THF: 5-metiltetrahidrofolato (Adaptado de Lentz, 2005).

O fígado é o principal órgão de degradação do excesso de Met e manutenção dos níveis adequados de Hcy. A SAM desempenha um papel central na regulação dos níveis de Hcy. Quando os níveis de SAM estão elevados, há uma redução na via de remetilação da Hcy à Met através da inibição da atividade da metileno tretrahidrofolato redutase (MTHFR). Por outro lado, quando ocorre o acúmulo de Hcy, a SAM promove o catabolismo desse aminóacido através da transulfuração por ativação da CBS (Finkelstein, 2007).

2. Hiper-homocisteinemia

No plasma, a Hcy circula em diferentes formas. Cerca de 70% está associada com proteínas plasmáticas, principalmente albumina, como dissulfetos mistos com resíduos de cisteína das proteínas. Aproximadamente 5 a 10% se encontra na forma de dímeros de Hcy (homocistina) ou dímeros homocisteína-cisteína através de uma ponte dissulfeto e 1 a 2% como Hcy livre. A Hcy plasmática total é a soma das formas livre, dímeros e ligada a proteínas (Welch and Loscalzo, 1998).

Em indivíduos normais, níveis plasmáticos de Hcy estão entre 5 a 15 µmol/L. A elevação dos níveis de Hcy, causada por fatores genéticos e ambientais, gera uma condição chamada de hiper-homocisteinemia que pode ser classificada em três graus conforme a severidade: leve (16-30 µmol/L), moderada (31-100 µmol/L) e severa (> 100 µmol/L) (Banecka-Majkutewicz et al., 2012). A hiper-homocisteinemia severa é rara e pode ser causada por deficiência genética na atividade da enzima CBS caracterizando a homocistinúria, uma desordem autossômica recessiva que pode ser gerada por 92 diferentes mutações no gene que codifica a enzima CBS. A homocistinúria clássica apresenta uma prevalência mundial que varia de 1:200.000 a 1:335.000 nascidos vivos e resulta no acúmulo de Hcy e Met nos tecidos e plasma (Mudd et al., 2001). Ao contrário da hiper-homocisteinemia severa que é encontrada principalmente em pacientes com doenças genéticas raras, as hiper-homocisteinemias moderada e leve são prevalentes na população, desde que podem ser causadas pelo polimorfismo no gene da enzima MTHFR, deficiências nutricionais de ácido fólico, vitaminas B_6 e B_{12}, ingestão

aumentada de Met, insuficiência renal crônica, e em decorrência do uso de certos medicamentos como, por exemplo, metotrexato, L-dopa, fenitoína e carbamazepina (Aguilar et al., 2004; Lentz, 2005). Pacientes afetados pelo acúmulo de Hcy apresentam manifestações clínicas que afetam o sistema ocular, ósseo, cardiovascular e sistema nervoso central (SNC) (Mudd et al., 2001).

2.1. Modelos animais de hiper-homocisteinemia

Têm sido descritos na literatura vários modelos experimentais para a indução da hiper-homocisteinemia. A hiper-homocisteinemia severa pode ser induzida pelo uso de animais *nocaute* para a enzima CBS (Watanabe et al., 1995), com deficiência severa na atividade da enzima MTHFR (Chen et al., 2001), ou ainda ser induzida quimicamente em animais (Streck et al., 2002). Em 2002 foi desenvolvido no nosso grupo de pesquisa o modelo experimental de hiper-homocisteinemia severa para mimetizar a homocistinúria. Nesse modelo, os animais recebem injeções subcutâneas de Hcy do 6º ao 28º dia de vida, período caracterizado por um rápido desenvolvimento cerebral em ratos, sendo que os níveis plasmáticos de Hcy chegam a 500 µM, semelhante aos pacientes portadores de homocistinúria (Streck et al., 2002). Usando esse modelo, nosso grupo tem mostrado, ao longo de dez anos, várias alterações neuroquímicas e comportamentais em ratos, tais como estresse oxidativo em cérebro, sangue (Matte et al., 2009a), coração (Kolling et al., 2011), fígado (Matte et al., 2009b) e pulmão (da Cunha et al., 2011), alterações nas atividades das enzimas da cadeia transportadora

de elétrons (Streck et al., 2003), Na^+,K^+-ATPase (Machado et al., 2011), acetilcolinesterase (da Cunha et al., 2012), inflamação (da Cunha et al., 2010) e déficit na memória (Matte et al., 2007).

Para a indução das hiper-homocisteinemias moderada e leve são utilizados animais com deficiência genética heterozigótica na CBS (Dayal et al., 2004) ou na MTHFR (Chen et al., 2001) e principalmente intervenções dietéticas que afetam o metabolismo da Hcy via transulfuração ou remetilação (Blaise et al., 2007). A deficiência de vitamina B_6, por exemplo, limita o fluxo do aminoácido via transulfuração enquanto que a deficiência de vitamina B_{12} ou ácido fólico prejudica a remetilação da Hcy à Met. A hiper-homocisteinemia também tem sido experimentalmente induzida pela adição de grandes quantidades de Met ou Hcy à ração ou água dos animais, ou através de uma combinação de dietas contendo grandes quantidades de Met associada à baixa quantidade de ácido fólico e vitamina B_{12}. Esses modelos animais são desenvolvidos em mini-porcos (Rolland et al., 1995), macacos (Lentz et al., 1997), coelhos (Sauls et al., 2007), camundongos (Dayal and Lentz, 2007) e ratos (Becker et al., 2005; De Vriese et al., 2004). Porém, alguns estudos mostram resultados controversos o que pode ser atribuído, pelo menos em parte, à escolha do modelo experimental de hiper-homocisteinemia (Dimitrova et al., 2002). Para o nosso conhecimento, há carência de modelos de hiper-homocisteinemia leve quimicamente induzida em animais.

2.2. Hiper-homocisteinemia leve como fator de risco para doenças cerebrais e cardiovasculares

Uma variedade de estudos mostram que a hiper-homocisteinemia leve é um fator de risco para doenças cerebrais e cardíacas tais como as doenças de Alzheimer (Minagawa et al., 2010) e Parkinson (dos Santos et al., 2009), acidente vascular cerebral, aterosclerose e tromboembolismo venoso (Abbracchio et al., 2006; Den Heijer et al., 2005; Wald et al., 2002). Além disso, elevados níveis de Hcy também têm sido associados a outras desordens que afetam o SNC, tais como depressão (Permoda-Osip et al., 2013) e esquizofrenia (Wysokinski and Kloszewska, 2013). Neste contexto, um estudo prévio mostra que uma elevação de cerca de 5 µmol/L nos níveis plasmáticos de Hcy está associada com um risco 20% maior para o desenvolvimento de doenças cardiovasculares (Chan et al., 2002). Santos e cols. (2009) demonstraram que os níveis de Hcy são 30% maiores em plasma de pacientes com doença de Parkinson em relação a indivíduos normais.

Os mecanismos pelos quais a Hcy exerce seus efeitos tóxicos não estão completamente elucidados. No cérebro, a Hcy tem sido associada à morte neuronal via excitotoxicidade, através da ativação de receptores glutamatérgicos metabotrópicos do grupo I (Zieminska et al., 2003) e ionotrópico N-metil-*D*-aspartato (NMDA) (Lipton et al., 1997). Além disso, a Hcy pode sofrer auto-oxidação do seu grupo tiol e causar prejuízo à homeostase redox, danificando células vasculares e neuronais (Perna et al., 2003; Weiss et al., 2003; Zou and Banerjee, 2005). Foi demonstrado que a Hcy aumenta a neurotoxicidade do peptídeo beta amilóide por indução de estresse oxidativo

(Ho et al., 2001). Outro mecanismo proposto é que a Hcy causa prejuízo nos processos de metilação celular. O acúmulo de Hcy leva a um aumento na SAH, um potente inibidor de reações de metilação que são vitais para a função neurológica como metilação de aminas biogênicas, da mielina e síntese de fosfatidilcolina (Troen, 2005).

Em relação aos danos vasculares, a Hcy reduz a biodisponibilidade de óxido nítrico ($NO^{\bullet}$) e aumenta a adesão e agregação de plaquetas estimulando a formação de trombos. Além disso, esse aminoácido também atua sobre alguns fatores estimulando as vias de coagulação e promovendo a oxidação do colesterol LDL (Julve et al., 2013; Stanger et al., 2004; Upchurch et al., 1997; Welch and Loscalzo, 1998).

3. Estresse Oxidativo

3.1. Radicais Livres

Radical livre pode se definido como qualquer espécie química que contém um ou mais elétron(s) desemparelhado(s) no seu orbital mais externo. O elétron desemparelhado geralmente leva a uma elevada reatividade da molécula (Halliwell and Gutteridge, 2007). As principais espécies reativas podem ser divididas em dois grupos: espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN). As ERO mais importantes são o ânion superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio (H_2O_2), radical hidroxila ($OH^{\bullet}$), ânion hipoclorito (OCl^-) e o oxigênio “singlet” ($^1O_2$). ERO são geradas nos sistemas vivos através do metabolismo energético celular. A redução tetravalente completa do oxigênio molecular (O_2) na cadeia transportadora de elétrons

mitocondrial é essencial para a formação de água. No entanto, aproximadamente 5% do O_2 não é completamente reduzido à água nesse processo, sendo convertido a $O_2^{\bullet}$, H_2O_2 e $OH^{\bullet}$. O $NO^{\bullet}$ e o peroxinitrito ($ONOO^-$), formado a partir da reação do $NO^{\bullet}$ com o $O_2^{\bullet-}$, constituem as principais ERN (Halliwell and Whiteman, 2004; Valko et al., 2004).

ERO e ERN podem ser benéficas ou deletérias para sistemas vivos. Em baixas concentrações atuam, por exemplo, na defesa contra agentes infecciosos e nos processos de sinalização intracelular. Por outro lado, elevadas concentrações de espécies reativas causam danos às biomoléculas como lipídios, proteínas e DNA e podem levar a perda de função celular. A peroxidação da bicamada lipídica presente nas membranas celulares altera a fluidez e prejudica a seletividade para a troca iônica, além de liberar produtos como o malondialdeído e o 4-hidroxinonenal que são potencialmente tóxicos. As proteínas podem ser oxidadas pelas ERO, o que pode prejudicar a atividade de enzimas, proteínas transportadoras e receptores. As espécies reativas também podem oxidar bases púricas e pirimídicas levando a mutações no DNA (Halliwell, 2012; Valko et al., 2007).

As espécies reativas diferem em relação à reatividade: o $O_2^{\bullet-}$ é pouco reativo não sendo capaz de promover peroxidação lipídica. Sua reatividade é devida principalmente à reação com outras espécies reativas como o $NO^{\bullet}$ para formar $ONOO^-$, geração de H_2O_2 e formação de $OH^{\bullet}$ através da reação de Haber-Weiss. O H_2O_2 por si só não é um radical livre e também não promove peroxidação lipídica direta (Halliwell and Gutteridge, 2007). No entanto, ele é capaz de gerar o $OH^{\bullet}$ através das reações de Fenton na presença de íons Cu^{+2} ou Fe^{+2}. Diferentemente do $O_2^{\bullet-}$ e H_2O_2, o $OH^{\bullet}$ tem uma elevada reatividade,

tornando-se uma espécie reativa muito perigosa com uma meia-vida muito curta (aproximadamente 10^{-9}s) (Pastor et al., 2000). Essa ERO pode iniciar o processo de peroxidação lipídica e reagir com outras biomoléculas. O $ONOO^-$ é capaz de depletar antioxidantes e promover dano a lipídios, proteínas e DNA (Alvarez and Radi, 2003; Halliwell and Whiteman, 2004; Halliwell, 2006).

3.2. Defesas Antioxidantes

A exposição a radicais livres, a partir de uma variedade de fontes, levou o organismo ao desenvolvimento de uma série de mecanismos de defesa. Dentre eles, podemos destacar as defesas antioxidantes enzimáticas e não enzimáticas. As defesas antioxidantes enzimáticas incluem a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx). Os antioxidantes não-enzimáticos são representados pelo ácido ascórbico (vitamina C), α-tocoferol (vitamina E), GSH, carotenóides, flavonóides e outros antioxidantes (Halliwell and Gutteridge, 2007; Halliwell, 2011).

A SOD é uma metaloenzima que catalisa a reação de dismutação do ânion $O_2^{\bullet-}$, formando H_2O_2 e O_2. Existem três isoformas dessa enzima: a cobre/zinco dependente (CuZnSOD), a manganês dependente (Mn/SOD) e a ferro dependente (Fe/SOD). A CuZn/SOD é encontrada no citosol, lisossomos, espaço mitocondrial intermembranas e peroxissomos. Existe ainda outro tipo de CuZn/SOD, chamada de SOD extracelular (EC-SOD), presente em fluidos extracelulares. A Mn/SOD é encontrada nas mitocôndrias enquanto a Fe/SOD é encontrada em bactérias (Yu, 1994).

O H_2O_2 pode ser decomposto pelas enzimas CAT e GPx. A CAT atua na decomposição do H_2O_2 a H_2O e O_2 sendo encontrada principalmente nos

peroxissomos da maioria dos tecidos em humanos, estando presente em maior quantidade no fígado. Por outro lado, o cérebro apresenta pequenas quantidades de CAT (Marklund et al., 1982). A GPx, localizada nas membranas celulares, decompõe o H_2O_2 através do acoplamento de sua redução a H_2O com a concomitante oxidação da GSH ao dissulfeto de glutationa (GSSG) ($H_2O_2 + 2GSH \rightarrow GSSG + 2\ H_2O$) (Halliwell and Gutteridge, 2007).

Em condições fisiológicas, existe um equilíbrio entre a produção de espécies reativas e as defesas antioxidantes. Entretanto, quando esse equilíbrio é rompido ocorre o estresse oxidativo que pode estar associado a diversas patologias. Todas as células aeróbias sofrem danos oxidativos, no entanto, o cérebro dos mamíferos é altamente sensível. Alguns fatores que tornam o SNC altamente suscetível às espécies reativas incluem: alto consumo de oxigênio; presença de neurotransmissores que sofrem auto-oxidação como dopamina, serotonina e noradrenalina; alta concentração de ferro; lipídios de membrana ricos em ácidos graxos insaturados e defesas antioxidantes modestas (Halliwell, 2006; Halliwell, 2012).

O estresse oxidativo também desempenha um papel relevante em doenças que afetam o sistema cardiovascular como aterosclerose, doença cardíaca isquêmica, hipertensão e insuficiência cardíaca congestiva (Kukreja and Hess, 1992; Misra et al., 2009; Nediani et al., 2011). Alterações como hipertrofia, modificações na contratilidade e apoptose de células cardíacas têm sido atribuídas ao aumento na produção de espécies reativas no coração (Giordano, 2005).

4. Na^+,K^+-ATPase

A Na^+,K^+-ATPase, primeira bomba de íons a ser identificada, é uma proteína integral de membrana que simultaneamente transloca 3 íons Na^+ para o compartimento extracelular em troca do transporte para dentro da célula de 2 íons K^+. A energia necessária para a troca iônica é derivada da hidrólise de uma molécula de ATP. A Na^+,K^+-ATPase funcional é composta por duas subunidades α e duas subunidades menores β. A subunidade α, responsável pela atividade catalítica da enzima, sofre fosforilação e transição conformacional acoplada à hidrólise de ATP e transporte dos íons Na^+ e K^+. Essa subunidade também contém o sítio de ligação da ouabaína (glicosídeo cardíaco e inibidor específico da enzima). A subunidade β é uma proteína glicosilada de adesão intercelular necessária para direcionar a subunidade α para a membrana plasmática. Além disso, estudos mostram a presença de outra subunidade ainda menor (γ) que regula a atividade da enzima (Geering, 2008) (Figura 2).

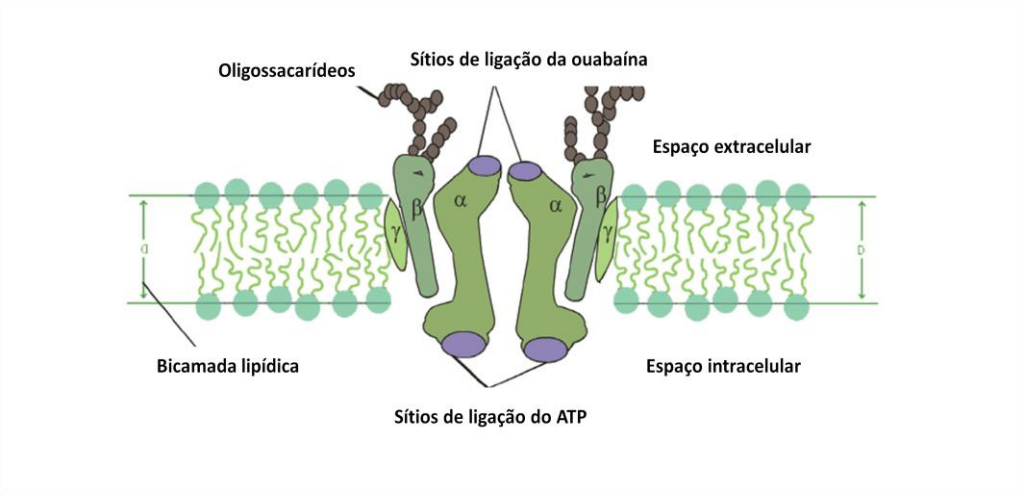

Figura 2: Estrutura da Na^+,K^+-ATPase (Adaptada de Suhail, 2010)

Foram identificados em mamíferos genes que codificam quatro isoformas da subunidade catalítica α (α_1, α_2, α_3 e α_4). As isoformas α_1, α_2, α_3 se encontram em diferentes tipos de células do SNC, entretanto a α_4 não é expressa no cérebro. A isoforma α_1 é ubiquamente expressa enquanto a α_2 é expressa em pequenos subconjuntos de neurônios e em muitos astrócitos. A isoforma α_3 é expressa exclusivamente em neurônios de várias estruturas cerebrais como hipocampo, cerebelo e gânglios da base (Bottger et al., 2011; McGrail et al., 1991). Existem três isoformas da subunidade β em mamíferos (β_1, β_2 e β_3). No cérebro, tem sido sugerido que a isoforma β_1 é expressa em neurônios enquanto a β_2 é encontrada em astrócitos (Benarroch, 2011; Geering, 2008). Além das subunidades funcionais α e β, uma subunidade γ modulatória, pertencente à família das proteínas FXYD foi encontrada no cérebro. Entretanto, essa subunidade não é essencial para a atividade da Na^+, K^+-ATPase (Geering, 2008).

A força do gradiente de íons Na^+ e K^+ gerado pela enzima conduz múltiplas funções celulares tais como regulação do volume celular, pH, manutenção do potencial de membrana em repouso e transporte de moléculas ligadas ao co-transporte de Na^+, como aminoácidos, neurotransmissores e glicose (Blanco, 2005; Jorgensen et al., 2003). As funções das diferentes isoformas da subunidade catalítica da Na^+,K^+-ATPase têm sido investigadas. Nos neurônios, a restauração dos gradientes de Na^+ e K^+ pela isoforma α_3 é muito importante para a manutenção da excitabilidade neuronal e condução do potencial de ação em axônios mielinizados e para o transporte secundário de neurotransmissores acoplados ao Na^+ (Benarroch, 2011). Em astrócitos, a α_2 é co-localizada com diferentes transportadores de glutamato, gerando o

gradiente eletroquímico necessário para a captação desse neurotransmissor e consequente finalização da sinalização glutamatérgica (Rose et al., 2009).

Nos últimos anos, estudos têm demonstrado que doses de ouabaína, insuficientes para inibir a atividade da enzima, modulam a proliferação celular, apoptose, interação célula-célula e migração celular. Esses efeitos ocorrem por ligação da ouabaína a Na^+,K^+-ATPase, reforçando o conceito de que enzima pode funcionar como um transdutor de sinal independente do bombeamento de íons (Aperia, 2007; Desfrere et al., 2009).

A Na^+,K^+-ATPase pode ser regulada por diversos fatores como disponibilidade de substrato, componentes da membrana celular, hormônios e fosforilação (Lopina, 2000; Wang and Yu, 2005). Além disso, essa enzima é suscetível ao ataque de radicais livres (Wang et al., 2003), sendo inibida por metabólitos formados durante a peroxidação lipídica e por alterações na membrana plasmática (Chakraborty et al., 2003; Dencher et al., 2007; Rauchova et al., 1999).

Modificações na atividade da Na^+,K^+-ATPase têm sido associadas a diversas patologias que afetam o SNC tais como enxaqueca (Suhail, 2010), doença de Alzheimer (Vitvitsky et al., 2012), depressão (Goldstein et al., 2006), Parkinson e epilepsia (Aperia, 2007; Benarroch, 2011). Além disso, estudos prévios realizados em nosso grupo de pesquisa mostraram que alguns aminoácidos como a Met (Stefanello et al., 2011), prolina (Ferreira et al., 2011) e a Hcy, em concentrações similares às encontradas na homocistinúria (~500 μM) (Machado et al., 2011) inibem a atividade da Na^+,K^+-ATPase cerebral provavelmente através da indução de estresse oxidativo, uma vez que a administração de antioxidantes foi capaz de prevenir tais alterações.

5. Inflamação

A inflamação é um mecanismo homeostático complexo desenvolvido para proteger a integridade do organismo contra agentes nocivos endógenos ou exógenos. A resposta inflamatória envolve células e fatores solúveis, e consiste na sequencial liberação de mediadores e o recrutamento de leucócitos da circulação, que se tornam ativados no local da inflamação liberando mais mediadores. Entre esses mediadores se destacam as proteínas de fase aguda (proteína C reativa, fibrinogênio, alfa-1-glicoproteína ácida, alfa-1-antitripsina, haptoglobina e ceruloplasmina), citocinas (interleucina 1-β (IL-1β), interleucina 2 (IL-2), interferon gamma (IFN-γ) e fator de necrose tumoral alfa (TNF-α)), quimiocinas (interleucina 8 (IL-8) e proteína quimiotática de monócito do tipo 1 (MCP-1)) prostaglandinas (PGE_2) e $NO^{\bullet}$ (Colton, 2009; Salerno et al., 2002; Zhang, 2008).

Outros mediadores têm sido investigados no processo inflamatório. Entre esses, pode-se destacar os nucleotídeos e nucleosídeo de adenina (ATP e Ado), bem com as enzimas envolvidas no controle dos seus níveis extracelulares (ectonucleotidases) (Bours et al., 2006). Além disso, estudos demonstram o papel da acetilcolina (ACh) na interação do sistema nervoso com o sistema imune inato para controlar a resposta inflamatória (Rosas-Ballina and Tracey, 2009).

A inflamação está presente em várias doenças neurodegenerativas e cardiovasculares. Um aumento nos níveis de proteínas inflamatórias foi observado em cérebro e plasma de pacientes com doença de Alzheimer e demência vascular (Engelhart et al., 2004). As citocinas pró-inflamatórias,

produzidas por macrófagos ativados e linfócitos T, têm sido associadas à esquizofrenia (Smith and Maes, 1995; Tourjman et al., 2013). Além disso, estudos mostram que as citocinas inflamatórias desempenham um papel muito importante na patogênese da aterosclerose (Libby and Ridker, 2004; Wolf et al., 2013).

5.1. Citocinas

As citocinas são um grupo de proteínas solúveis de baixo peso molecular, sintetizadas e secretadas por diversos tipos de células mediante estímulos provenientes de situações fisiológicas ou patológicas. Elas são produzidas principalmente por monócitos, macrófagos, células endoteliais, linfócitos e fibroblastos (Hirano, 1992). Além disso, também podem ser produzidas e secretadas dentro do SNC, pela microglia e pelos astrócitos (Jones and Thomsen, 2013). Essas proteínas medeiam a sinalização célula-célula, se ligam a receptores de superfície de alta afinidade, podem ter ação local ou sistêmica e influenciar a síntese de outras citocinas (Bruunsgaard, 2005).

As citocinas pró-inflamatórias TNF-α, IL-1β e interleucina 6 (IL-6) desempenham papel crucial na resposta de fase aguda. O TNF-α, secretado por macrófagos, linfócitos e monócitos (Vitale and Ribeiro Fde, 2007), é um potente ativador de neutrófilos. Além disso, essa citocina estimula as células endoteliais a expressar moléculas de adesão e promove alterações na permeabilidade vascular (Hehlgans and Pfeffer, 2005). A IL-1β é produzida por macrófagos, neutrófilos, células epiteliais e endoteliais em resposta a outras

citocinas, como o TNF-α, ou produtos bacterianos como o lipopolissacarídeo (LPS). Ela atua sobre o endotélio aumentando a expressão de moléculas de adesão (Cao et al., 2005; Garlanda et al., 2013; Kondera-Anasz et al., 2005). A IL-6, produzida em resposta ao TNF-α, à IL-1β e a algumas células T ativadas, estimula a síntese de proteínas de fase aguda pelo fígado, promove proliferação de linfócitos B e secreção de anticorpos (Hirota et al., 2005).

A Hcy ativa células endoteliais em cultura, o que resulta no aumento da expressão de quimiocinas e moléculas de adesão (Koga et al., 2002; Silverman et al., 2002). Poddar e cols. (2001) demonstraram que concentrações de 10 a 50 μM de Hcy promovem um estado pró-inflamatório com o aumento da expressão e secreção de IL-8 e MCP-1 em cultura de células endoteliais aórticas humanas. Além disso, a hiper-homocisteinemia severa crônica aumentou a concentração das citocinas pró-inflamatórias (TNF-α, IL-1β e IL-6), e da quimiocina CCL_2 (MCP-1) em hipocampo e soro de ratos neonatos (da Cunha et al., 2012).

5.2. ATP e Adenosina

Muitas evidências mostram o importante papel do ATP e da Ado na imunidade e inflamação. Concentrações extracelulares de ATP no microambiente de células danificadas podem aumentar consideravelmente, marcando o local danificado e contribuindo para a iniciação da resposta imune. A Ado, por outro lado, parece ter um importante papel imunossupressor (Blackburn et al., 2009; Bours et al., 2006; Di Virgilio et al., 2009).

O ATP é reconhecido como um neurotransmissor e neuromodulador no SNC. Ele é co-liberado com outros neurotransmissores como glutamato, noradrenalina, ACh e serotonina e age através de dois tipos de purinoreceptores: P1 e P2 (Burnstock, 2004). Os receptores P1 são ativados principalmente por Ado enquanto os P2 são ativados por ATP (Abbracchio et al., 2006). Estudos mostram que o ATP exerce um papel pró-inflamatório através da ativação de receptores $P2X_7$, induzindo a produção de citocinas como IL-1β, IL-2, IL-12, IL-18 e TNF-α por células do sistema imunológico (Bours et al., 2006; Solle et al., 2001).

A Ado é um modulador que exerce a sua ação através de quatro tipos de receptores metabotrópicos - A1, A2A, A2B e A3 (Fredholm et al., 2001). Em particular, a ação anti-inflamatória dessa molécula ocorre pela ligação ao receptor A2A encontrado no endotélio vascular, monócitos, macrófagos, neutrófilos, mastócitos, linfócitos, plaquetas e neurônios (Blackburn et al., 2009; Sullivan, 2003). A ativação do receptor A2A pela Ado controla a produção de mediadores inflamatórios como IL-12 e TNF-α pelas células imunológicas e inibe a ativação de linfócitos T (Erdmann et al., 2005; Hasko and Cronstein, 2013; Lappas et al., 2005).

É importante destacar que o metabolismo intracelular da Hcy e da Ado estão intimamente relacionados. O equilíbrio da reação catalisada pela SAHH, que hidrolisa SAH em Ado e Hcy, favorece a formação de SAH. Entretanto, a reação é dirigida no sentido oposto pela rápida remoção de Hcy e Ado, fazendo com que a enzima catalise a reação de clivagem de SAH. Entretanto, quando ocorre um aumento nos níveis de Hcy, há um consumo de Ado e acúmulo de SAH. Sob tais circunstâncias, a formação de Ado intracelular é prejudicada,

aumentando o gradiente de concentração transmembrana que existe em condições normais. Desse modo, a concentração extracelular de Ado é diminuída através da captação pelo transportador equilibrativo de nucleosídeo bidirecional (Riksen et al., 2003).

6. Ectonucleotidases

Nucleotídeos extracelulares são hidrolisados pelas ectonucleotidases, que podem estar localizadas na superfície celular, no meio intersticial ou na forma solúvel (Zimmermann, 2001). Dentre elas, pode-se destacar a família das ecto-nucleosídeo trifosfato difosfoidrolases (E-NTPDases), ecto-nucleotídeo pirofosfatase/fosfodiesterases (E-NPPs), ecto-5'-nucleotidases e fosfatases alcalinas. As E-NTPDases hidrolisam nucleotídeos trifosfatados e difosfatados (ATP e difosfato de adenosina (ADP)) a monofosfatados (monofosfato de adenosina (AMP), as quais se diferenciam quanto à afinidade por substratos. Oito diferentes genes codificam os membros da família das E-NTPDases: NTPDase 1, 2, 3 e 8 estão localizadas na superfície celular; NTPDase 5 e 6 exibem localização intracelular ou podem ser secretadas, enquanto as NTPDases 4 e 7 são intracelularmente localizadas, voltadas para o lúmen de organelas citoplasmáticas (Longhi et al., 2013; Yegutkin, 2008) (Figura 3).

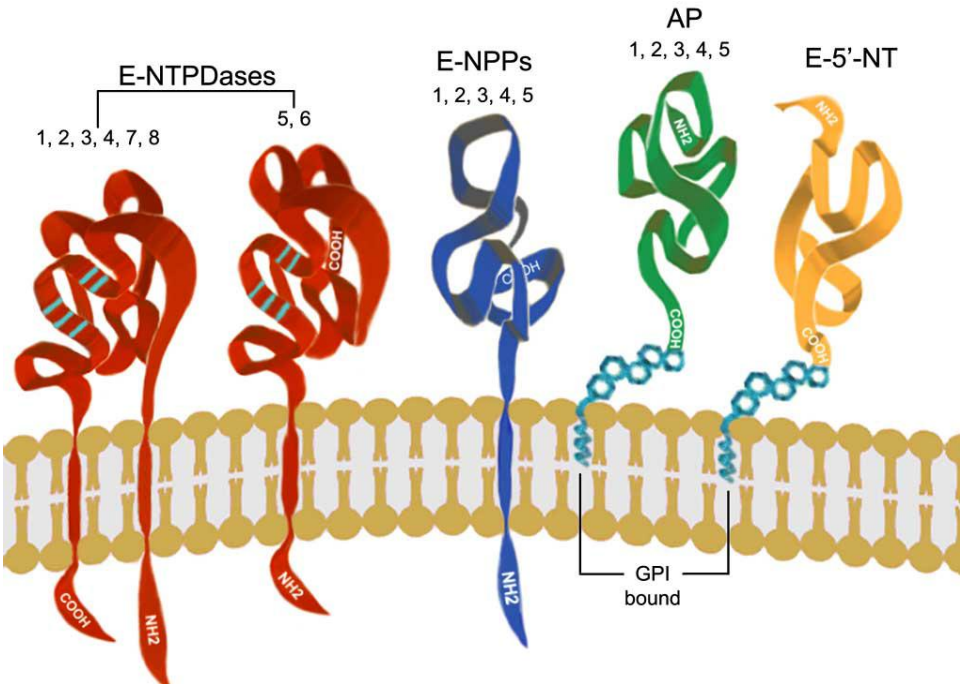

Figura 3: Enzimas que degradam nucleotídeos extracelulares. E-NPTDases e E-NPPs são enzimas integrais de membrana, enquanto que as fosfatases alcalinas (AP) e a ecto-5'-nucleotidase (5'-NT) estão ancoradas à membrana por um resíduo de glicosil fostafitidil inositol (GPI) (adaptado de Cognato e Bonan, 2010).

A NTPDase 1, o primeiro membro da família das E-NTPDases, foi identificada como um antígeno de ativação celular (CD39), um marcador de ativação de células B (Maliszewski et al., 1994; Yegutkin, 2008). Essa enzima é expressa, entre outros, em células vasculares (Robson et al., 2005), células dendríticas (Mizumoto et al., 2002) e linfócitos (Deaglio et al., 2007). A razão de hidrólise de ATP e ADP pela NTPDase 1 é 1:1. A NTPDase 2 está presente principalmente em células da glia e hidrolisa 30 vezes mais o ATP do que o ADP. A NTPDase 3 é principalmente associada a estruturais neuronais como axônios e sua preferência pela hidrólise de ATP em relação ao ADP é de 3:1 (Langer et al., 2007; Robson et al., 2006; Zimmermann, 2001).

A ecto-5'-nucleotidase, também conhecida como CD73, consiste de duas subunidades glicoprotéicas com aproximadamente 60 a 70 KDa

ancoradas à membrana plasmática por uma molécula de glicosil fosfatidil inositol (GPI). Essa enzima hidrolisa AMP a Ado sendo expressa de forma variável nos tecidos, com expressão abundante no cólon, rim, cérebro e coração (Moriwaki et al., 1999; Thompson et al., 2004; Zimmermann, 1996). Em linfócitos T e B, a expressão da enzima está correlacionada com a maturação celular, enquanto neutrófilos, eritrócitos e plaquetas expressam pouca enzima (Yegutkin, 2008). A ação coordenada das ectonucleotidases é muito importante para a regulação dos níveis de nucleotídeos e Ado em condições fisiológicas ou patológicas (Al-Rashida and Iqbal, 2013).

Dados demonstram que a Hcy modula a atividade das ectonucleotidases. A administração aguda de Hcy em ratos (0,6 µmol/g de peso corporal) aumentou a hidrólise de nucleotídeos em hipocampo e soro (Bohmer et al., 2004). Concentrações de 5 mM e 8 mM de Hcy inibiram a hidrólise de ATP, ADP e AMP em soro de ratos (Bohmer et al., 2006). Zanin e cols. (2010) demonstraram que a Hcy (*in vitro* e *in vivo*) inibiu a hidrólise de ATP e ADP em plaquetas de ratos. Além disso, em macrófagos, foi demonstrado que esse aminoácido induz aumento na hidrólise de nucleotídeos. A Ado gerada foi provavelmente captada pelas células, uma vez que a formação do nucleosídeo inosina foi diminuída em macrófagos de ratos (Zanin et al., 2013).

7. Colinesterases

Neurônios colinérgicos e as suas projeções são amplamente distribuídos por todo o SNC desempenhando um papel essencial na regulação de várias funções vitais, como aprendizagem, memória, organização cortical do

movimento, e controle do fluxo sanguíneo cerebral. Em particular, a enzima acetilcolinesterase (AChE) é uma das enzimas chave do sistema colinérgico e é frequentemente usada como um marcador do seu funcionamento. Ela desempenha um papel central na regulação da concentração de ACh, sendo encontrada no organismo nas formas ligada à membrana e solúvel, e em maior concentração no SNC, membrana de eritrócitos e junção neuromuscular (Zimmerman and Soreq, 2006). Outra enzima que hidrolisa ACh é a butirilcolinesterase (BuChE). Essa enzima difere da AChE quanto à distribuição tecidual, sendo encontrada principalmente no soro, e na afinidade por substratos e inibidores (Giacobini, 2004; Massoulie et al., 1993). Embora o papel fisiológico dessa enzima não esteja completamente elucidado, sabe-se que a atividade da BuChE é muito importante na manutenção da integridade do sistema colinérgico (Mesulam et al., 2002).

Estudos têm sugerido o envolvimento da ACh na inflamação através da chamada “via anti-inflamatória colinérgica”, composta pelo nervo vago, pela ACh e pela subunidade α7 do receptor nicotínico de ACh. Essa via representa um mecanismo de resposta do SNC à presença de estímulos inflamatórios na circulação sendo mediada pela ação do nervo vago (Pavlov and Tracey, 2005; Pavlov and Tracey, 2012; Rosas-Ballina and Tracey, 2009; Tracey, 2007). Citocinas pró-inflamatórias como a IL-1β ativam as fibras aferentes do nervo vago, as quais servem de sensor para a inflamação (Gwilt et al., 2007; Pavlov and Tracey, 2005). Esta informação é transmitida ao SNC, o qual estimula o nervo vago eferente para a produção de ACh, que induz então a inibição da síntese e liberação de citocinas pró-inflamatórias por macrófagos e outras células produtoras de citocinas (Gwilt et al., 2007; Parrish et al., 2008). Assim,

a via colinérgica anti-inflamatória representa um mecanismo fisiológico pelo qual o SNC interatua com o sistema imune inato para controlar a resposta inflamatória (Gallowitsch-Puerta and Pavlov, 2007).

II. OBJETIVOS

2.1. Objetivo Geral

A fim de melhor compreender os mecanismos envolvidos nas alterações cerebrais e cardíacas promovidas pela hiper-homocisteinemia leve, o objetivo geral desse trabalho foi desenvolver um modelo químico experimental de hiper-homocisteinemia leve e investigar os efeitos da Hcy sobre parâmetros bioquímicos em ratos adultos submetidos a esse modelo.

2.2. Objetivos específicos:

Os objetivos específicos estão subdivididos em seis capítulos, que serão apresentados na forma de artigos científicos, como segue:

- **Capítulo I**

- Desenvolver um modelo animal químico experimental de hiper-homocisteinemia leve em ratos adultos;
- Investigar parâmetros de estresse oxidativo, denominados oxidação do DCFH, níveis de nitritos, potencial antioxidante total não enzimático (TRAP), substâncias reativas ao ácido tiobarbitúrico (TBARS), atividade das enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx), em sangue e córtex cerebral de animais submetidos ao modelo experimental de hiper-homocisteinemia leve.

- **Capítulo II**

- Dosar os níveis de Hcy em coração de ratos submetidos ao modelo experimental de hiper-homocisteinemia leve;

- Investigar parâmetros de estresse oxidativo, tais como oxidação do DCFH, níveis de nitritos, TRAP, conteúdo de GSH, atividade das enzimas antioxidantes (SOD e CAT), TBARS, conteúdo de sulfidrilas e de carbonilas em coração e aorta de animais hiper-homocisteinêmicos;

➢ **Capítulo III**

- Avaliar a atividade da Na^+,K^+-ATPase em córtex cerebral e hipocampo de ratos hiper-homocisteinêmicos;
- Investigar o efeito da Hcy sobre o imunoconteúdo das subunidades catalíticas (isoformas α_1, α_2 e α_3) da Na^+,K^+-ATPase em córtex cerebral e hipocampo de ratos;
- Analisar, em córtex cerebral e hipocampo, a expressão gênica das subunidades catalíticas (isoformas α_1, α_2 e α_3) da enzima.

➢ **Capítulo IV**

- Investigar o efeito da hiper-homocisteinemia leve sobre a hidrólise de nucleotídeos de adenina (ATP, ADP e AMP) em linfócitos e soro de ratos;
- Avaliar a expressão das ectonucleotidases em linfonodos mesentéricos de ratos adultos submetidos à administração crônica de Hcy.

➢ **Capítulo V**

- Verificar o efeito da administração crônica de Hcy sobre a hidrólise de ATP, ADP e AMP em sinaptossomas obtidos de córtex cerebral de ratos;

- Avaliar a expressão das ectonucleotidases em córtex cerebral de ratos adultos submetidos ao modelo experimental de hiper-homocisteinemia leve;
- Dosar os níveis de ATP, ADP, AMP e Ado em líquor de ratos hiper-homocisteinêmicos;

➢ **Capítulo VI**

- Investigar os níveis das citocinas (TNF-α, IL-1β e IL-6) e da quimiocina CCL_2 (MCP-1) no córtex cerebral, hipocampo, coração e soro de ratos submetidos à hiper-homocisteinemia leve crônica;
- Avaliar o efeito da Hcy sobre a atividade da enzima AChE em córtex cerebral e hipocampo de ratos;
- Estudar o imunoconteúdo da AChE em córtex e hipocampo de ratos hiper-homocisteinêmicos;
- Investigar o efeito da Hcy sobre a expressão da AChE em córtex e hipocampo de ratos;
- Avaliar a atividade da enzima BuChE em soro de ratos submetidos ao modelo experimental de hiper-homocisteinemia leve.

III. METODOLOGIA E RESULTADOS

Os capítulos I, II, III, IV, V e VI serão apresentados na forma de artigos científicos, os quais apresentam o mesmo desenho experimental de hiper-homocisteinemia leve crônica.

3.1. Modelo experimental de hiper-homocisteinemia leve crônica

Ratos Wistar foram submetidos a duas injeções subcutâneas diárias de Hcy (0,03 µmol/g de peso corporal) do 30º ao 60º dia de vida. Os animais foram mortos 12 h após a última administração do aminoácido. Animais controle receberam o mesmo volume de solução salina.

Capítulo I

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ABSTRACT

The purpose of this study was to develop a chronic chemically induced model of mild hyperhomocysteinemia in adult rats. We produced levels of Hcy in the blood (30 μM), comparable to those considered a risk factor for the development of neurological and cardiovascular diseases, by injecting homocysteine subcutaneously (0.03 μmol/g of body weight) twice a day, from the 30th to the 60th postpartum day. Controls received saline in the same volumes. Using this model, we evaluated the effect of chronic administration of homocysteine on redox status in the blood and cerebral cortex of adult rats. Reactive oxygen species and thiobarbituric acid reactive substances were significantly increased in the plasma and cerebral cortex, while nitrite levels were reduced in the cerebral cortex, but not in the plasma, of rats subjected to chronic mild hyperhomocysteinemia. Homocysteine was also seen to disrupt enzymatic and non-enzymatic antioxidant defenses in the blood and cerebral cortex of rats. Since experimental animal models are useful for understanding the pathophysiology of human diseases, the present model of mild hyperhomocysteinemia may be useful for the investigation of additional mechanisms involved in tissue alterations caused by homocysteine.

1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that is metabolized by remethylation to methionine or by transsulfuration to cysteine via cystathionine (Williams and Schalinske, 2010). Plasma total Hcy concentrations in the 5–15 μM range are considered to be normal in humans. Severe hyperhomocysteinemia (>100 μM) results from homozygous genetic defects, such as a deficiency of cystathionine-β-synthase (CβS), methylene-tetrahydrofolate reductase (MTHFR) or of enzymes involved in methyl-B_{12} synthesis and Hcy methylation. Mild (15–30 μM) and moderate (31–100 μM) hyperhomocysteinemia can be caused by vitamin deficiencies, heterozygous CβS defect, the thermolability of MTHFR or environmental causes such as ageing and gender, impaired renal function, smoking, alcohol consumption, stress and others (Castro et al., 2006; De Bree et al., 2002; Jacques et al., 2001; Selhub, 2006; Troen et al., 2008). Although severe hyperhomocysteinemia is rare, mild and moderate hyperhomocysteinemia are common in many populations since they are influenced by lifestyle factors and can be secondary to systemic diseases or drug treatment (De Bree et al., 2002).

During the last decade, epidemiological studies have shown that a mild plasma Hcy concentration is a risk factor for cerebral (Blaise et al., 2007; Kado et al., 2005; Mattson and Shea, 2003) and cardiovascular diseases (De Bree et al., 2002; Huang et al., 2008; Sharma et al., 2008). Moreover, hyperhomocysteinemia has been reported to be a potential risk factor for a number of other pathologies, including diabetes, hypertension, psoriasis, birth defects, and cancer (Cakmak et al., 2009; Kim, 1999; Lin et al., 2010; Steegers-Theunissen et al., 1994; Tessari et al., 2005; Tyagi et al., 2005a).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism. ROS and RNS are well recognized for playing dual roles as both deleterious and favorable species, since they can be either harmful or beneficial to living systems (Valko et al., 2006). Reactive species include molecules such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), nitric oxide ($NO^{\bullet}$), and lipid radicals. On the other hand, hydrogen

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peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl) are not free radicals per se, but have oxidizing effects. Under physiological conditions, a series of defense mechanisms act to maintain the balance between production and elimination of reactive species. Enzymatic antioxidant defenses include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Non-enzymatic antioxidants are represented by ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids, and others (Halliwell and Gutteridge, 2007). However, when the pro-oxidant/antioxidant balance is disturbed towards higher concentrations of ROS/RNS by increased production and/or decreased antioxidant protection, a phenomenon called oxidative stress may occur, which can negatively affect cellular components. Based on this, it has been suggested that oxidative stress can play a role in the pathogenesis of several neurodegenerative and cardiac diseases (Halliwell, 2006; Nediani et al., 2010).

Experimental animal models can be used to better understand the pathophysiology of human diseases. In this context, our laboratory has developed animal models for several inherited metabolic disorders, such as hyperprolinemia type II (Moreira et al., 1989), phenylketonuria (Wyse et al., 1994, 1995), methylmalonic and propionic acidemias (Brusque et al., 1999; Dutra et al., 1991), severe hyperhomocysteinemia (homocystinuria) (Streck et al., 2002) and hypermethioninemia (Stefanello et al., 2007).

Mild experimental hyperhomocysteinemia has been commonly induced by diet models, such as folate/vitamin B_{12} depletion or high methionine (Blaise et al., 2007; Fukada et al., 2006). Although these models are of much interest to study the pathogenesis of hyperhomocysteinemia, it has been proposed that the effects may not be due to elevated Hcy, but due to abnormalities induced by reduction in folate or elevation in methionine (Perna et al., 1996; Hansrani and Stansby, 2008). In addition, both folate deficiency and methionine excess have been proposed to have adverse effects on vascular function that may be independent of elevated Hcy (Doshi et al., 2002; Troen et al., 2003). Based on these considerations and the fact that, to our knowledge, there are no studies on the induction of chronic mild hyperhomocysteinemia in adult rats by subcutaneous Hcy administration; in the present study our main objective was to develop a chemically induced chronic experimental model of mild hyperhomocysteinemia in adult rats. Using this model, we evaluated some parameters of oxidative damage in the blood and cerebral cortex of hyperhomocysteinemic rats.

2. Materials and methods

2.1. Animals and reagents

Wistar rats (30 and 60 days old) were obtained from the Central Animal House of the Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in an air conditioned constant room temperature (22 ± 1 °C) colony, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985) and was approved by the University Ethical Committee (#19634).

2.2. Chemically induced mild hyperhomocysteinemia

Wistar rats (60 days old) received a subcutaneous injection of Hcy (0.03 μmol/g of body weight). Animals were subsequently killed by decapitation without anesthesia and the blood and cerebrum were removed at 0, 15, 30, 60, 120 min and 12 h after Hcy injection for Hcy level determination. The blood was centrifuged at 1000 × g and the plasma was separated. The cerebrum was immediately isolated, washed with saline solution; the olfactory bulbs, pons, medulla and the cerebellum were discarded and the cerebrum was homogenized in 2 volumes of saline solution and centrifuged at 1000 × g. Doses of Hcy administered were chosen in order to induce plasma levels of 15–30 μM, similar to those considered a risk factor for developing cerebral and cardiovascular diseases. We then determined the following pharmacokinetic parameters, plasma half-time ($t_{1/2}$), apparent volume of distribution (V_d) and plasma clearance (Cl_p) after Hcy injection.

2.3. Homocysteine level determination

Hcy levels in plasma and cerebrum were determined as described by Magera et al. (1999), using liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS). After samples reduction and deproteinization, the Hcy concentration was detected through the transition from the precursor to the product ion (*m/z* 136 to *m/z* 90). Homocysteine-d (8) was added as an internal standard.

2.4. Chronic mild hyperhomocysteinemia

For the chronic, chemically induced mild hyperhomocysteinemia, Hcy (0.03 μmol/g of body weight) was administered subcutaneously from the 30th to the 60th day of life. Control rats received saline solution in the same volumes (0.5 mL/100 g of body weight). The animals were killed by decapitation 15, 30, 60 min and 12 h after the last injection for Hcy level determination in plasma and cerebrum, as described above. For evaluating oxidative stress parameters, the animals were killed by decapitation, 12 h after the last injection of Hcy and the blood and cerebral cortex were removed.

2.5. Erythrocytes and plasma preparation

Whole blood was collected and transferred to heparinized tubes for erythrocyte separation. Blood samples were centrifuged at 1000 × g, plasma was removed by aspiration and frozen at −80 °C until determination. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride). Lysates were prepared by the addition of 1 mL of distilled water to 100 μL of washed erythrocytes and frozen at −80 °C until determination of the antioxidant enzyme activities. For antioxidant enzyme activity determination, erythrocytes were frozen and thawed three times to break the erythrocyte membrane and release enzymes, and centrifuged at 13,500 × g for 10 min. The supernatant was diluted in order to contain approximately 0.5 mg/mL of protein.

2.6. Cerebral cortex preparation

The cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Afterwards, the homogenate was centrifuged at 800 × g for 10 min at 4 °C, and the supernatant was retained for biochemical assays.

2.7. 2′7′-Dichlorofluorescein (H_2DCF) oxidation assay

Reactive oxygen species production was measured following the protocol of LeBel et al. (1990), based on the oxidation of 2′7′-dichlorofluorescein (H_2DCF). Plasma or cortex supernatants (60 μL) were incubated for 30 min at 37 °C in the dark with 240 μL of 100 μM 2′7′-dichlorofluorescein diacetate (H_2DCF-DA) solution in a 96-well plate. H_2DCF-DA is cleaved by cellular esterases and the resultant H_2DCF is eventually oxidized by ROS present in samples. The latter reaction produces the fluorescent compound, dichlorofluorescein (DCF), which was measured at 488 nm excitation and 525 nm emission and the results were represented by nmol DCF/mg protein.

2.8. Thiobarbituric acid reactive substances (TBARS)

TBARS, an index of lipid peroxidation, was determined according to the method described by Ohkawa et al. (1979). For determination, plasma and cortex supernatants in 1.15% KCl were mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS were determined by the absorbance at 535 nm and calculated as nmol of malondialdehyde (MDA) formed per milligram of protein.

2.9. Determination of nitrite levels

Nitrite is the stable endproduct of the autoxidation of NO• in aqueous solution (Ignarro et al., 1993). Nitrite levels were measured using the Griess reaction; 100 μL of plasma and cortex supernatants were mixed with 100 μL Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature. The absorbance was measured on a microplate reader at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards (Green et al., 1982).

2.10. Superoxide dismutase assay (SOD)

The SOD activity assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm (Marklund, 1985). A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were reported as units

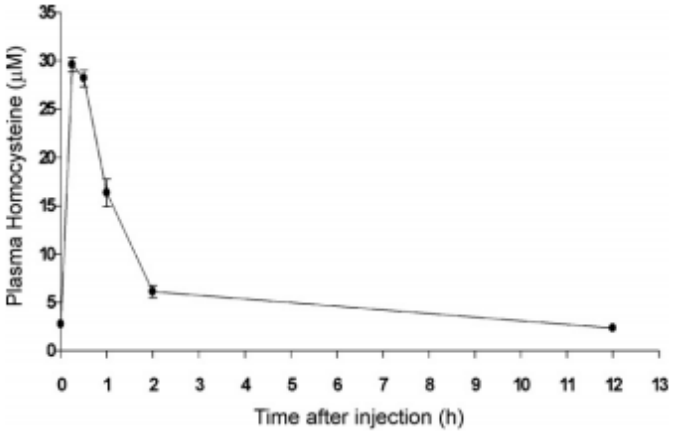

Fig. 1. Homocysteine plasma levels. Homocysteine was administered to rats of 60 days of age and the blood was collected at 0, 15, 30, 60, 120 min and 12 h after injection. Data are expressed as means ± S.D. for 3–4 animals per group.

per milligram of protein. SOD activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit.

2.11. Catalase assay (CAT)

CAT activity was assayed using a double-beam spectrophotometer with temperature control (Hitachi U-2001). This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL (Aebi, 1984). One CAT unit is defined as 1 mmol of H_2O_2 consumed per minute and the specific activity is represented as CAT units/mg protein.

2.12. Glutathione peroxidase (GPx)

GPx activity was measured by the method of Wendel (1981), except for the concentration of NADPH, which was adjusted to 0.1 mM after previous tests performed in our laboratory. Tert-butylhydroperoxide was used as substrate. NADPH disappearance was continuously monitored with a spectrophotometer at 340 nm for 4 min. One GPx unit is defined as 1 mmol of NADPH consumed per minute and specific activity is reported as units/mg protein.

2.13. Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) thermolysis (Lissi et al., 1992; Evelson et al., 2001) with a Perkin-Elmer Microbeta Microplate Scintillation Analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Two-hundred and forty microliters of a system containing ABAP (10 mM), dissolved in 50 mM sodium phosphate buffer pH 8.6, and luminol (5.6 mM), was added to a microplate, and the initial chemiluminescence was measured. Ten microliters of 300 μM Trolox (standard) or 10 μL of plasma and cortex supernatants were then added to each plate well, producing a decrease in the initial chemiluminescence value. This value is kept low, until the antioxidants present in the sample are depleted, the chemiluminescence then returns to its initial value. The time taken by the sample to keep chemiluminescence low is directly proportional to the antioxidant capacity of the tissue. The results were represented as nmol Trolox/mg protein.

2.14. Protein determination

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.15. Statistical analysis

Data were analyzed by Student's *t*-test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Chemically induced mild hyperhomocysteinemia

Fig. 1 shows that in time between 15 and 30 min after Hcy injection (0.03 μmol/g of body weight) plasma Hcy levels were approximately 30 μM, similar to those described in the plasma of patients with mild hyperhomocysteinemia (De Bree et al., 2002). Twelve hours after Hcy administration, the plasma levels of this amino acid returned to normal values. Hcy was not detected in the cerebrum, probably due to the sensitivity of the method (1 μM).

Table 1
Pharmacokinetic parameters and dose administered of homocysteine.

Age (day)	$t_{1/2}$ (hours)	V_d (mL/g)	Cl_p (mL/h/g)	Hcy dose (μmol/g weight)
60	4.8	1.82	0.31	0.03

Note: The parameters were calculated after an acute subcutaneous injection of Hcy, after which animals were sacrificed at regular time intervals (0, 15, 30, 60, 120 min and 12 h); $t_{1/2}$ = plasma half-time; V_d = apparent volume of distribution; Cl_p = plasma clearance.

The pharmacokinetic parameters, plasma half-time ($t_{1/2}$), apparent volume of distribution (V_d) and plasma clearance (Cl_p) were determined after Hcy injection (Table 1). Table 2 shows that chronically Hcy-treated rats presented a peak plasma level of 34 μM at 15 min after the last injection of this amino acid, returning to normal values at 12 h after the last injection. Taking these data into consideration, we induced levels of Hcy comparable to those considered a risk factor for cardio- and cerebrovascular diseases by subcutaneously injecting a buffered Hcy solution twice a day, from the 30th to the 60th day of life.

The body (control: 168 ± 9.7; Hcy: 170 ± 8.9), cerebrum (control: 1.66 ± 0.07; Hcy: 1.68 ± 0.09), and heart (control: 0.63 ± 0.06; Hcy: 0.67 ± 0.06) weights of rats submitted to chronic Hcy administration did not differ from those of saline-treated rats, suggesting that Hcy did not cause malnutrition in the animals.

3.2. Effect of chronic mild hyperhomocysteinemia on parameters of oxidative stress in blood of rats

Table 3 shows that Hcy significantly increased the levels of ROS measured by DCF formed from the oxidation of H_2DCF [$t(8) = -2.708$; $p < 0.05$] and increased TBARS in the plasma of rats [$t(10) = -2.495$; $p < 0.05$], as compared to the control.

Table 2
Plasma homocysteine levels after chronic administration.

Group	Plasma homocysteine levels (μM)
Saline	3.0 ± 0.25
Hcy – 15 min	34.8 ± 0.94
Hcy – 30 min	31.7 ± 0.15
Hcy – 60 min	19.3 ± 3.01
Hcy – 12 h	3.93 ± 1.88

Note: Hcy was administered from the 30th to the 60th day of life. Control rats received saline solution in the same volumes. The blood was collected 15, 30, 60 min and 12 h after the last injection. Data are expressed as mean ± S.D. for 3–4 rats in each group.

Table 3
Parameters of oxidative stress in blood of hyperhomocysteinemic rats.

	Control	Homocysteine
Plasma		
DCF (nmol/mg protein)	49.85 ± 3.64	55.79 ± 3.27*
TBARS levels (nmol MDA/mg protein)	3.25 ± 0.28	3.97 ± 0.65*
Nitrite levels (μmol/L)	20.1 ± 2.01	23.65 ± 4.00
TRAP (nmol Trolox/mg protein)	15.85 ± 2.44	12.37 ± 1.20*
Erythrocytes		
SOD/CAT ratio	0.65 ± 0.06	0.90 ± 0.1**
GPx activity (units/mg protein)	22.96 ± 1.47	23.77 ± 2.68

* Significantly different from control ($p < 0.05$).
** Significantly different from control ($p < 0.01$).

However, the nitrite levels were not affected by Hcy administration [$t(7) = -1.615$; $p > 0.05$].

The activities of antioxidant enzymes (SOD, CAT and GPx) were evaluated in erythrocytes of hyperhomocysteinemic rats. Results show a significant increase in the SOD/CAT ratio in erythrocytes of Hcy-treated rats [$t(10) = -4.855$; $p < 0.01$]. As reported in Table 3, Hcy did not change GPx activity [$t(10) = -0.652$; $p > 0.05$].

We also investigated the status non-enzymatic antioxidant defenses in plasma of rats. Hcy administration significantly reduced TRAP [$t(8) = -2.852$; $p < 0.05$], as compared to control (Table 3).

3.3. *Effect of chronic mild hyperhomocysteinemia on parameters of oxidative stress in the cerebral cortex of rats*

Reactive oxygen species, TBARS and nitrite levels in the cerebral of cortex of adult rats were evaluated. Fig. 2 shows a significant increase in DCF levels (A) [$t(8) = -4.251$; $p < 0.01$] and TBARS (B) [$t(10) = -6.365$; $p < 0.001$]. Nitrite levels were significantly decreased in the cerebral cortex of hyperhomocysteinemic rats [$t(9) = 4.392$; $p < 0.01$] (Fig. 3).

The effect of chronic mild hyperhomocysteinemia on antioxidant enzymes (SOD, CAT and GPx) in the cerebral cortex was also evaluated. Fig. 4 shows that Hcy significantly increased SOD/CAT ratio (A) [$t(8) = -3.202$; $p < 0.05$]; however, GPx activity was not affected by Hcy administration [$t(10) = -0.529$; $p > 0.05$]. Next, we determined the antioxidant potential in the cerebral cortex of rats subjected to chronic mild hyperhomocysteinemia. Fig. 5 shows that Hcy administration significantly reduced TRAP [$t(7) = 6.332$; $p < 0.001$], as compared to the control.

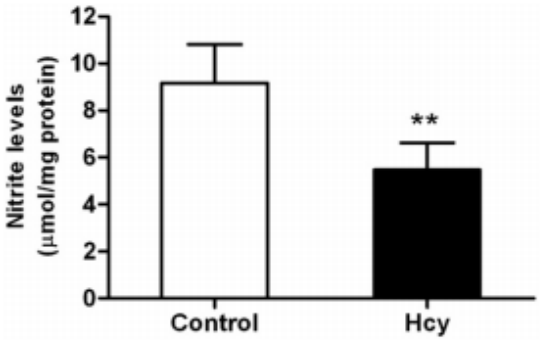

Fig. 3. Effect of chronic homocysteine administration on nitrite levels in the cerebral cortex of rats. Results are expressed as mean ± S.D. for 5–6 animals per group. Different from control, $^{**}p < 0.01$ (Student's *t*-test). Hcy: homocysteine.

4. Discussion

Since elevated levels of circulating Hcy increase the risk for developing coronary artery disease, peripheral vascular disease, myocardial infarction, stroke and multiple neurological disorders, such as Alzheimer's disease, age-related dementias and Parkinson's disease (Blaise et al., 2007; Manolescu et al., 2010; Obeid and Herrmann, 2006), the main objective of the present study was to develop an experimental model of chronic mild hyperhomocysteinemia in adult rats, whose plasma levels of Hcy were similar to those considered a risk factor for neurological and cardiovascular diseases.

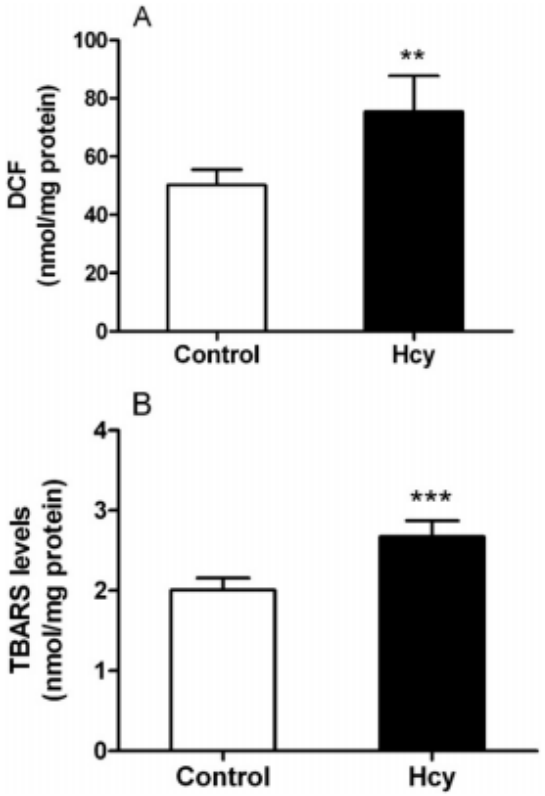

Fig. 2. Effect of chronic homocysteine administration on free radical levels (DCF formed) (A) and thiobarbituric acid reactive substances (TBARS) (B) in the cerebral cortex of rats. Results are expressed as mean ± S.D. for 5–6 animals per group. Different from control, $^{**}p < 0.01$; $^{***}p < 0.001$ (Student's *t*-test). Hcy: homocysteine.

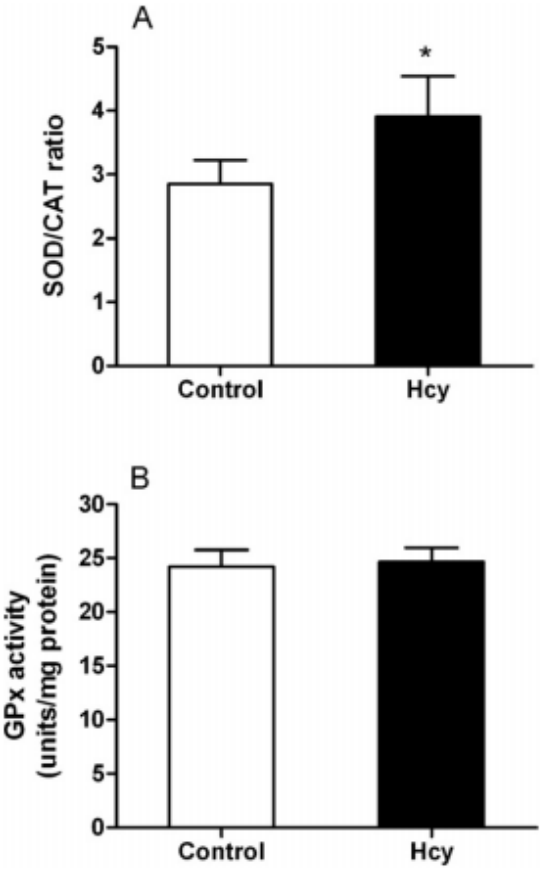

Fig. 4. Effect of chronic homocysteine administration on superoxide dismutase/catalase ratio (A) and glutathione peroxidase activity (B) in the cerebral cortex of rats. Results are expressed as mean ± S.D. for 5–6 animals per group. Different from control, $^{*}p < 0.05$ (Student's *t*-test). Hcy: homocysteine.

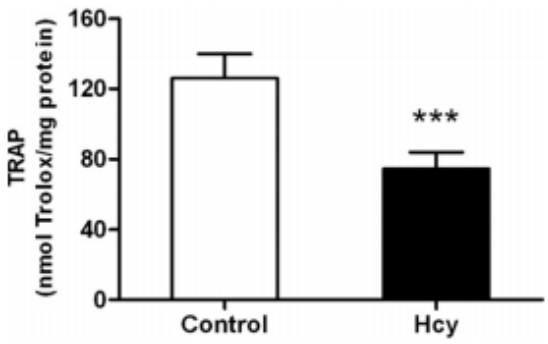

Fig. 5. Effect of chronic homocysteine administration on total radical-trapping antioxidant potential (TRAP) in the cerebral cortex of rats. Results are expressed as mean ± S.D. for 4–5 animals per group. ***$p < 0.001$ (Student's *t*-test). Hcy: homocysteine.

In our study the rats subjected to mild hyperhomocysteinemia present concentrations of approximately 30 μM of Hcy in the plasma between 15 and 30 min after subcutaneous administration of this amino acid, returning to normal values 12 h after a single injection of Hcy. From this study, we determined the doses that induced chronic mild hyperhomocysteinemia, observed from the 30th to the 60th day of life of the animals. The peak plasma concentrations of Hcy were similar to those found after acute administration. Our findings demonstrated that animals undergoing this treatment have no differences in body, cerebrum or heart weight, when compared to the control group, indicating that Hcy does not cause malnutrition in the rats. In the cerebrum, Hcy concentrations were not detected, probably due to the sensitivity of the method (1.0 μM); however, we have shown previously that, at higher plasma concentrations, Hcy (400–500 μM) crosses the blood–brain barrier (Streck et al., 2002). Moreover, it has been reported that the neurotoxic effects of Hcy may be associated with its uptake by membrane transporter and consequent intracellular accumulation (Grieve et al., 1992).

Although an association between Hcy and oxidative stress has been suggested (Mattson and Shea, 2003; Ventura et al., 2009), the mechanisms by which Hcy causes cerebral and cardiovascular diseases remain poorly understood. The cerebrum may be particularly vulnerable to high levels of this amino acid because it lacks one metabolic pathway for Hcy elimination: betaine remethylation and the transsulfuration pathway is incomplete (Finkelstein, 1998). Moreover, the central nervous system is sensitive to oxidative stress, mainly due to its high oxygen consumption, low antioxidant levels, high iron and polyunsaturated fatty-acid side-chains contents (Halliwell and Gutteridge, 2007).

We have shown previously that chronic severe hyperhomocysteinemia, similar to that encountered in homocystinuric patients, alters antioxidant defenses in the brain and blood of rats (Matté et al., 2009). In the present study, we examined the effects of chronic mild hyperhomocysteinemia on some parameters of oxidative stress in the blood and cerebral cortex of adult rats. We observed an increase in the levels of reactive oxygen species and TBARS, an index of lipid peroxidation (Halliwell, 2001) in plasma and cerebral cortex of hyperhomocysteinemic rats. These results corroborate with studies demonstrating that during the auto-oxidation of Hcy reactive species may be generated, such as $O_2^{\bullet-}$, H_2O_2, or $OH^{\bullet}$, which have harmful effects for most cellular components including lipids, proteins, carbohydrates, and DNA (Nishio and Watanabe, 1997; Tyagi, 1998; Welch et al., 1998).

It has been reported that the oxidative effects of Hcy can cause perturbations in the vasculature. Experimental studies in both cerebral and peripheral blood vessels support the concept that oxidative stress caused by Hcy perturbs the delicate balance between the production and breakdown of $NO^{\bullet}$, a critical regulator of vascular homeostasis (Dayal and Lentz, 2005; Eberhardt et al., 2000; Stamler et al., 1993; Ungvari et al., 2003; Upchurch et al., 1997). Under normal conditions, $NO^{\bullet}$ plays a role in the detoxification of Hcy through the formation of *S*-nitrosohomocysteine (Stamler et al., 1993). However, chronic exposure to Hcy increases the formation of $O_2^{\bullet-}$, which can react with $NO^{\bullet}$ to yield the potent oxidant, $ONOO^{-}$, a powerful mediator of lipid peroxidation (Tyagi et al., 2005b; Zhang et al., 2000).

In our study, the nitrite concentration was unaltered in the plasma after administration of Hcy, but $NO^{\bullet}$ bioavailability may be reduced due to the generation of free radicals and lipid peroxidation caused by Hcy. Murawska-Cialowicz et al. (2008) have demonstrated an increase in lipid peroxidation in the blood of rats after the administration of a diet rich in methionine, without any change in the concentrations of $NO^{\bullet}$ metabolites (nitrites and nitrates). On the other hand, chronic mild hyperhomocysteinemia was seen to decrease nitrite levels in the cerebral cortex, suggesting that the spontaneous reaction of $NO^{\bullet}$ with thiol groups of the Hcy itself and/or $O_2^{\bullet-}$ may have reduced its availability in this cerebral structure.

Studies show that Hcy can regulate protein turnover and gene expression, including antioxidant enzymes (Sharma et al., 2006; Yamamato et al., 2000). In the present study, we also investigated the effect of chronic mild hyperhomocysteinemia on enzymatic antioxidant defenses in the blood and cerebral cortex of rats, which comprise an efficient system responsible for removing reactive species (Halliwell and Gutteridge, 2007). Animals exposed to chronic mild hyperhomocysteinemia were found to present an imbalance between SOD and CAT activities, expressed as an increased SOD/CAT ratio in erythrocytes and cerebral cortex, while the GPx activity was unchanged. SOD dismutates $O_2^{\bullet-}$ with the generation of H_2O_2, which is reduced by CAT and GPx. When a cell has decreased activities of these enzymes, a large amount of H_2O_2 becomes available to react with transition metals to form $OH^{\bullet}$, the most powerful free radical, through the Fenton and Haber–Weiss reactions.

TRAP is a useful estimate of non-enzymatic antioxidants present in a given biological fluid. Changes in TRAP have been observed in various clinical conditions, including ageing, acute infection, diabetes mellitus, and coronary heart disease (Alho and Leinonen, 1999). In the present study, we observed that Hcy reduced TRAP in the plasma of rats. Our results agree with those of Lee et al. (2007) who showed that hyperhomocysteinemia is associated with a decrease in the concentrations of antioxidant vitamins, such as vitamins A and E, and an increase in TBARS in the plasma of rats. In addition, *in vitro* studies have shown that Hcy added to the medium during incubation reduces TRAP in parietal and prefrontal cortices, suggesting a direct effect of this amino acid on non-enzymatic antioxidant defenses (Matté et al., 2004). Here, we found that the administration of Hcy also decreased TRAP in the cerebral cortex of adult rats, suggesting that this amino acid may compromise non-enzymatic antioxidant capacity.

Since the endothelial surface, lipoproteins in the plasma, as well as polyunsaturated fatty acid present in the brain membrane are particularly susceptible to $OH^{\bullet}$ attack (Gutteridge, 2001; Matés et al., 1999), the alterations in antioxidant defenses (enzymatic and non-enzymatic) observed in our study could contribute, at least in part, to the increase in ROS and lipid peroxidation observed in the plasma and cerebral cortex of rats subjected to chronic mild hyperhomocysteinemia.

In summary, we have developed a chemically induced chronic model of mild hyperhomocysteinemia in adult rats in order to investigate the possible mechanisms by which Hcy exerts toxicity. Using this model, we showed that Hcy increased

reactive oxygen species formation and lipid peroxidation, as well as decreased antioxidant defenses, suggesting an oxidative damage that could be associated with the physiopathology of disorders that accumulate Hcy. Considering that the incidence of mild hyperhomocysteinemia has grown steadily in the general population, the present experimental model may be useful in the investigation of additional mechanisms involved in the tissue alterations caused by Hcy.

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Capítulo II

Redox imbalance in heart and aorta from rats submitted to chronic mild hyperhomocysteinemia

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Redox imbalance in heart and aorta from rats submitted to chronic mild hyperhomocysteinemia

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Abstract

The aim of our study was to investigate some parameters of oxidative/nitrative stress in heart and aorta from rats subjected to mild hyperhomocysteinemia. We also measured the homocysteine levels in cardiac tissue. Homocysteine (0.03 μmol/g of body weight) or saline (control) were administered subcutaneously from the 30th to the 60th day of life of rats. Results show that homocysteine levels were increased in heart of rats subjected to hyperhomocysteinemia (5.3 μM at 30 min and 1.8 μM at 12 h after the last injection of homocysteine). Mild hyperhomocysteinemia decreased total radical-trapping antioxidant potential and increased protein carbonyl content in the heart of rats. In the aorta, homocysteine altered antioxidant defenses (decreased total radical-trapping antioxidant potential and increased superoxide dismutase activity), decreased nitrite levels and induced lipid and protein damage (increased thiobarbituric acid reactive species and carbonyl levels); reactive oxygen species, glutathione content and catalase activity were not changed. Our findings show that chronic mild hyperhomocysteinemia induces oxidative/nitrative imbalance in heart and aorta, suggesting that the oxidative stress can be associated to cardiovascular complications observed in the mild hyperhomocysteinemia.

Keywords: Homocysteine; Mild Hyperhomocysteinemia; Oxidative stress; Heart; Aorta

Introduction

Homocysteine (Hcy) is an amino acid formed by the demethylation of methionine or by its catabolism. After this, Hcy may undergo remethylation to methionine or can enter in the transsulfuration pathway to cysteine synthesis.[1] Elevated levels of Hcy (hyperhomocysteinemia) occurs when the plasma concentration of Hcy exceeds 15 µmol/L, and can result from nutritional deficiencies of folate, B_6 and B_{12} vitamins, high dietary methionine intake, reduced renal function, or prejudiced activity of enzymes involved in its metabolism.[2-4]

In 1969, McCully[5] was the first to associate Hcy with cardiovascular risk. Since then, several studies have demonstrated that a mild to moderate elevation of Hcy (15-50 µM) is an independent risk factor for vascular diseases.[6-14] It has been proposed that an increase of plasma Hcy level by about 5 µM enhances the prevalence of cardiovascular diseases by 20%.[15,16] Although the mechanisms responsible for producing hyperhomocysteinemia-induced vascular changes are unclear, some studies in experimental animals and humans support the concept that oxidative stress, characterized by an imbalance between the oxidant and antioxidant systems in favor of the oxidants,[17-18] could play a role in the development of vascular disturbances promoted by Hcy.[19-23]

Oxidative stress has been implied in the initiation and progression of various cardiovascular diseases including atherosclerosis, hypertension, and heart failure. Reactive oxygen species (ROS) may interact directly with cellular lipids, proteins, and DNA damaging both cardiac myocytes and vascular cells.[24] Some important heart proteins, such as ion channels, sarcoplasmic reticulum

calcium release channels, and myofilament proteins can be altered by ROS.[25] The enhanced production of ROS may lead to endothelial dysfunction and harm the muscle tone, blood flow, and control of inflammatory reactions promoted by vascular endothelium.[26,27]

Using an experimental model of chronic mild hyperhomocysteinemia in adult rats developed in our laboratory, we have reported that animals subjected to model presented oxidative stress in blood and cerebral cortex. We showed that Hcy concentration in plasma of rats achieved levels similar to those described in the plasma of patients with mild hyperhomocysteinemia (30 μM).[28] In the present study we extend our investigation to evaluate the effects of chronic mild hyperhomocysteinemia on some oxidative/nitrative stress parameters (DCFH oxidation, nitrite levels, superoxide dismutase and catalase activities, total radical-trapping antioxidant potential, reduced glutathione content, thiobarbituric acid reactive substances, sulfhydryl and carbonyl contents) in heart and aorta of adult rats decapitated at 12 hours after the last injection of Hcy. The levels of this amino acid also were measured in cardiac tissue.

Material and methods

Animals and reagents

Wistar rats (30 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air conditioned constant

temperature (22±1ºC) colony room, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the ‘‘Principles of Laboratory Animal Care’’ (NIH publication 85-23, revised 1996) and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (#19634).

Chronic mild hyperhomocysteinemia

DL-Hcy (0.03 µmol/g of body weight) was administered subcutaneously twice a day from the 30th to the 60th day of life of rats; controls rats received saline solution in the same volumes (0.5 ml/100 g of body weight). Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those described in the plasma of patients with mild hyperhomocysteinemia (30 µM). [28] The animals were killed by decapitation at 30 min and at 12 h after the last injection of Hcy and the heart and aorta were removed.

Homocysteine level determination in the cardiac tissue

Hcy levels in cardiac tissue were determined as described by Magera,[29] using liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS). After samples reduction and deproteinization, Hcy concentration was detected through the transition from the precursor to the product ion (m/z 136 to m/z 90). Homocysteine-d (8) was added as an internal standard. Hcy levels were measured at 30 min and 12 h after the last injection of this amino acid.

Tissue preparation to measure of oxidative stress

The heart and aorta from animals decapitated at 12 h after the last injection of Hcy were homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The homogenate was centrifuged at 800 x g for 10 min at 4ºC, and the supernatant was retained for biochemical assays.

2′7′ dichlorofluorescein (H_2DCF) oxidation assay

This assay was utilized to measure the ROS production. [30] Heart and aorta supernatants (60 µL) were incubated for 30 min at 37 °C in the dark with 240 µL of 100 µM 2′7′-dichlorofluorescein diacetate (H_2DCF-DA) solution in a 96-well plate. H_2DCF-DA is cleaved by cellular esterases and the resultant H_2DCF is eventually oxidized by ROS present in samples. The latter reaction produces the fluorescent compound, dichlorofluorescein (DCF), which was measured at 488 nm excitation and 525 nm emission and the results were represented by nmol DCF/ mg protein.

Determination of nitrite levels

Nitrite level, metabolite of Nitric oxide (NO), was measured using the Griess's reagent;[31] 100 µL of heart and aorta supernatants were mixed with 100 µL Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature. The absorbance was measured at a wavelength of 543 nm. Nitrite standards were utilized to calculate nitrite concentration.[32]

Superoxide dismutase assay (SOD)

The assay is based on the ability of pyrogallol to autoxidize in the presence of superoxide, a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm.[33] A calibration curve was performed with purified SOD as standard. The results were reported as units per milligram of protein. SOD activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit.

Catalase assay (CAT)

This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL.[34] One CAT unit is defined as 1 mmol of H_2O_2 consumed per minute and the specific activity is represented as CAT units/mg protein.

Total radical-trapping antioxidant potential (TRAP)

TRAP was measured by chemiluminescence intensity of luminol induced by 2,2'-azo-bis-(2-amidinopropane) (ABAP) thermolysis[35-36] utilizing a Perkin-Elmer Microbeta Microplate Scintillation Analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Two-hundred and forty microliters of a system containing ABAP (10 mM), dissolved in 50 mM sodium phosphate buffer pH 8.6, and luminol (5.6 mM), was added to a microplate, and the initial chemiluminescence was measured. Ten microliters of 300 µM Trolox (standard) or 10 µl of heart and aorta supernatants were then added to each plate well,

producing a decrease in the initial chemiluminescence value. This value is kept low, until the antioxidants present in the sample are depleted, the chemiluminescence then returns to its initial value. The time taken by the sample to keep chemiluminescence low is directly proportional to the antioxidant capacity of the tissue. The results were represented as nmol Trolox/mg protein.

Reduced glutathione (GSH) content

This method is based on the reaction of GSH with the fluorophore o-phthalaldehyde (OPT) after deproteinizing the samples.[37] Fluorescence was measured at 420 nm emission and 525 nm excitation in a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA). A calibration curve was performed with a commercial GSH solution, and the results were calculated as nmol GSH/mg protein.

Thiobarbituric acid reactive substances (TBARS)

TBARS, an index of lipid peroxidation, was determined according to the method described by Ohkawa. [38] For analyzes, heart and aorta supernatants in 1.15% KCl were mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS were determined by the absorbance at 535 nm and calculated as nmol TBARS/mg protein.

Sulfhydryl content

The oxidation of free thiols in the sample leads to the formation of disulfide bonds. [39] The sulfhydryl content is inversely correlated to oxidative damage to proteins. In the assay, the 5,5′ dithio-bis(2-nitrobenzoic acid) (DTNB), color reagent is not reduced by the thiols oxidized, generating a yellow derivative (TNB), read spectrophotometrically at 412 nm. The results were represented as nmol TNB/mg protein.

Protein carbonyl content

This parameter is based in the increase of carbonyl content in the presence of oxidatively modified proteins. Protein carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm.[40] Results were represented as protein carbonyl content (nmol/mg protein).

Protein determination

Protein was measured by the method of Lowry[41] using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by the Student's T test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

Results

Homocysteine levels determination in the heart

The levels of Hcy were determined in the cardiac tissue of adult rats subjected to chronic hyperhomocysteinemia, which are illustrated in table 1. Hcy-treated rats presented a tissue level of 5.3 μM at 30 min and 1.8 μM at 12 h after the last injection of this amino acid as compared to animal controls (0.9 μM Hcy). These results demonstrate that in the heart tissue, the homocysteine peak occurs at 30 min at the same time as in the plasma, as previously demonstrated by our research group.[28] But at 12 hours after the last injection, unlike from the plasma whose levels of the amino acid return to normal, the levels of Hcy are twofold higher in the heart tissue than control.

Effect of chronic mild hyperhomocysteinemia on parameters of oxidative/nitrative stress in the heart of adult rats

We investigated the effects of mild hyperhomocysteinemia on several parameters of oxidative stress in the heart of rats. Table II shows that Hcy-treated rats not present alteration in DCFH oxidation, nitrite levels and antioxidant defenses [SOD and CAT activities, and GSH content] (p >0.05), but we observed a significant decrease in total radical-trapping antioxidant potential (TRAP) that is considered a measured of non-enzymatic antioxidant defenses (p <0.05).

Biomolecules damage was also evaluated by TBARS levels, sulfhydryl and carbonyl content, which are measured of lipid peroxidation and protein damage, respectively. TBARS and sulfhydryl were not altered (p >0.05), while protein carbonyl content was significantly increased (p <0.01) in the heart of adult rats.

Effect of chronic mild hyperhomocysteinemia on parameters of oxidative/nitrative stress in the aorta of adult rats

Figure 1 shows that Hcy did not alter the levels of reactive oxygen species measured by DCF formed from the oxidation of H_2DCF (p >0.05), whereas nitrite levels were significantly reduced in the aorta of hyperhomocystinemic rats (p <0.01).

Activities of antioxidant enzymes (SOD and CAT) were evaluated in aorta of adult rats submitted to chronic mild hyperhomocysteinemia. Results represented in Figure 2 show that SOD activity was significantly increased by Hcy administration (p < 0.05) whereas CAT activity was not altered (p >0.05).

Next, we investigated the status non-enzymatic antioxidant defenses in the aorta of rats (Figure 3). Hcy administration significantly reduced TRAP (p <0.05) as compared to control. But GSH levels were not affected by hyperhomocysteinemia (p >0.05).

Damage to lipids was also assessed in the aorta, and Hcy promoted an increase in TBARS levels (p <0.01). In relation to the protein damage, results demonstrated that sulfhydryl content was not altered (p >0.05), whereas protein carbonyl content was significantly decreased by hyperhomocysteinemia (p < 0.01) (Figure 4).

Discussion

Risk factors such as hypertension, hypercholesterolemia, and diabetes favor the development of cardiovascular diseases.[42-44] Recently it has been suggested that hyperhomocysteinemia is regarded as an independent risk

indicator for cardiovascular events whose mechanisms are not well elucidated.[45-47] In the present study, our objective was to evaluate some parameters of oxidative/nitrative stress in heart and aorta from rats subjected to mild hyperhomocysteinemia. Hcy levels were measured in the heart at 30 min and 12 h after the last injection of amino acid. Results demonstrated the rats subjected to chronic mild hyperhomocysteinemia, decapitated 30 min after the last injection of amino acid, present cardiac levels of Hcy about to 6 times higher than the control group. These data shown that the peak of Hcy levels in heart are increased at the same time as in the plasma peak occurs, as previously demonstrated by our research group. [28] In contrast to plasma analyses, whose Hcy levels returned to normal values at 12 hours after the last injection of Hcy, in cardiac tissue they still remain high by 2-fold compared to control animals, suggesting that Hcy metabolism in the heart is slower than in plasma, leading to an accumulation for a longer period of time in the cardiac tissue.

Regarding to effect of hyperhomocysteinemia on oxidative/nitrative stress in the heart of adult rats, results demonstrated that the ROS production (DCF formed), nitrite levels, antioxidant enzyme activities, GSH content, TBARS and sulfhydryl contents were not altered. In contrast, TRAP was significantly reduced, whereas protein carbonyl content was increased in the heart of hyperhomocysteinemic rats.

TRAP measures non-enzymatic antioxidants present in the tissue. In the heart, intracellular antioxidants include the vitamins E, C, and β-carotene, ubiquinone, lipoic acid, urate and GSH.[25] The lack of effect of Hcy on GSH levels with reduction in TRAP is puzzling, however, it is not known whether

depletion of GSH occurs significantly in animals with moderate or mild hyperhomocysteinemia, since in such conditions are not occurs block in Hcy transsulfuration pathway, that lead to synthesis of the glutathione, such as cystathionine-β-synthase deficiency or deficiency of vitamin B_6.[48] Thus, it is probable that the TRAP is reduced due to a depletion of vitamins in cardiac tissue, since it has been demonstrated that the hyperhomocysteinemia is associated with a decrease in the concentrations of antioxidant vitamins, such as vitamins A and E in other tissues, such as plasma of rats.[49]

The number of carbonyl groups is correlated with protein damage caused by ROS. Carbonyls can be result of protein glycation by sugars, direct oxidation of amino acid side chains by reactive species, aldehydes binding to proteins, among other factors.[50-52] Furthermore, protein carbonylation can also occur by reaction with various reactive products generated during lipid peroxidation such as 4-hydroxynonenal, 2-propenal, and malondialdehyde.[53] In the present study, we observed that chronic mild hyperhomocysteinemia provoked a significant increase in protein carbonyl. Analyzing these results it is possible that during 30 days of treatment, oxidative changes have occurred in the heart, since oxidative damage to proteins that are present. However, the cardiac tissue has been able to adapt to such effects promoted by homocysteine since studies demonstrated that this structure is very resistant to oxidative modifications.[54]

On arterial level, ROS production was not altered but nitrite levels, a stable product of NO, were significantly decreased after the chronic Hcy treatment. It was also observed an increase in SOD activity, but we did not observed none alteration in CAT activity. In relation to non-enzymatic antioxidants, it was observed a decrease in TRAP without alteration in GSH

content. TBARS levels and protein carbonyl were significantly increased in aorta of hyperhomocysteinemic rats.

NO is a key regulator of endothelial function. Vascular NO relaxes blood vessels, prevents platelet aggregation, limits oxidation of low density lipoprotein (LDL) cholesterol, inhibits proliferation of vascular smooth muscle cells, and decreases the expression of pro-inflammatory genes that advance atherogenesis.[55,56] Our results showing that are agreement with several studies show that hyperhomocysteinemia reduces the production and/or bioavailability of endothelium-derived NO by several mechanisms.[57-61]Hcy promotes the oxidation of the endothelial nitric oxide synthase (eNOS) cofactor, tetrahydrobiopterin (BH4), resulting in the uncoupling of the eNOS enzyme and consequent decrease in the NO production.[62] Hcy is also known to increase asymmetrical dimethylarginine, an endogenous inhibitor of eNOS. Furthermore, Hcy indirectly decreases NO bioavailability by generating superoxide that rapidly reacts with NO to yield the potent oxidant, peroxynitrite.[63,64]

NO is neutral, hydrophobic and capable of traversing membranes, and superoxide is anionic at neutral pH. Based on this, it has been suggested that the peroxynitrite formation occurs predominantly close to the sites of superoxide formation.[65] In this context, the increase in SOD activity in the present study could also be a response to higher levels of radical superoxide, suggesting that this enzyme activity can be altered by protect the arterial vessel of damage caused by this free radical, since the smooth muscle cells and the endothelial cells have a nicotinamide adenine dinucleotide phosphate oxidase, an enzyme that is placed at the membrane of these cells and is a strong source of superoxide anion.[66]

In the present study the Hcy increased SOD activity without increasing CAT, and it is known that the balance in these enzymes is also needed by oxidative cellular protection. SOD dismutates superoxide anion generating hydrogen peroxide, which when present in transition metals can yield the hydroxyl radical through Fenton and Habber-Weiss reactions.[18] Hydroxyl can react with several molecules and cause, as a consequence, damage in nucleic acids, proteins, carbohydrates, and lipids. The lipid peroxidation and protein damage observed in this study can be consequent of imbalance between the enzymatic and non-enzymatic antioxidant defenses that can generate powered free radicals such as peroxynitrite and hydroxyl radical.

In summary, the results of this study show that Hcy could be accumulated in cardiac tissue and that stress oxidative/nitrative are associated with deleterious effects of Hcy on the cardiovascular system.

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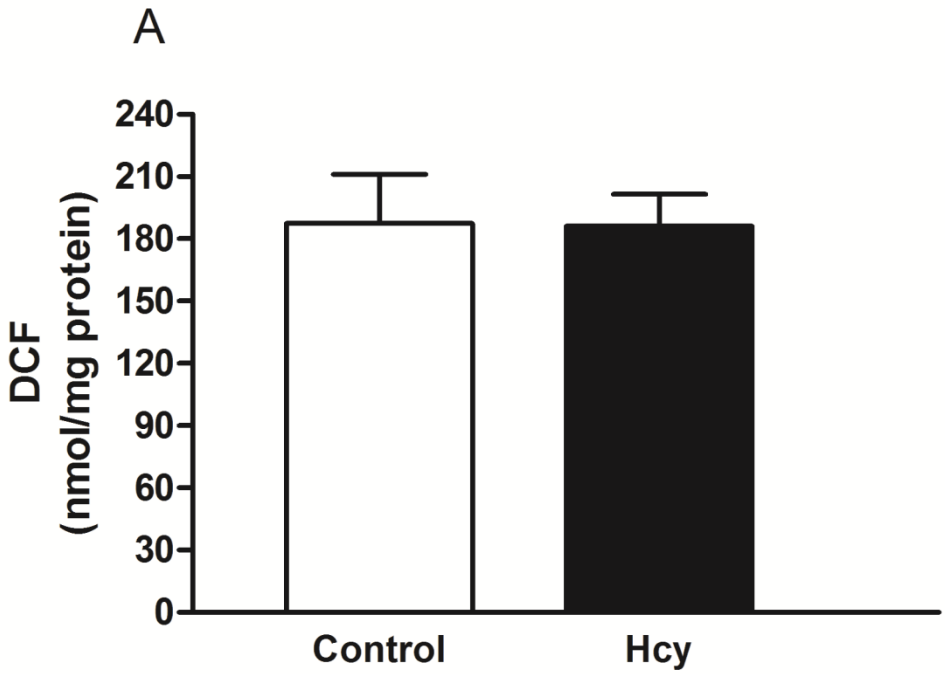
Legends to Figures

Figure 1. Effect of chronic homocysteine administration on reactive oxygen species (DCF formed) (A) and nitrite levels (B) in the aorta of rats. Results are expressed as mean ± S.D. for 5-8 animals in each group. Different from control, **p< 0.01 (Student's *t* test).

Figure 2. Effect of chronic homocysteine administration on superoxide dismutase (A) and catalase (B) activities in the aorta of rats. Results are expressed as mean ± S.D (n= 5-8 per group) for independent experiments performed in duplicate. Different from control, *p< 0.05 (Student's *t* test).

Figure 3. Effect of chronic homocysteine administration on total radical-trapping antioxidant potential (TRAP) (A) and GSH levels (B) in the aorta of rats. Results are expressed as mean ± S.D. for 5-8 animals in each group. Different from control, *p< 0.05 (Student's *t* test).

Figure 4. Effect of mild hyperhomocysteinemia on TBARS (A), sulfhydryl content (B) and protein carbonyl content (C) in the aorta of rats. Results are expressed as mean ± S.D (n= 5-8 per group) for independent experiments performed in duplicate. Different from control, **p< 0.01 (Student's *t* test).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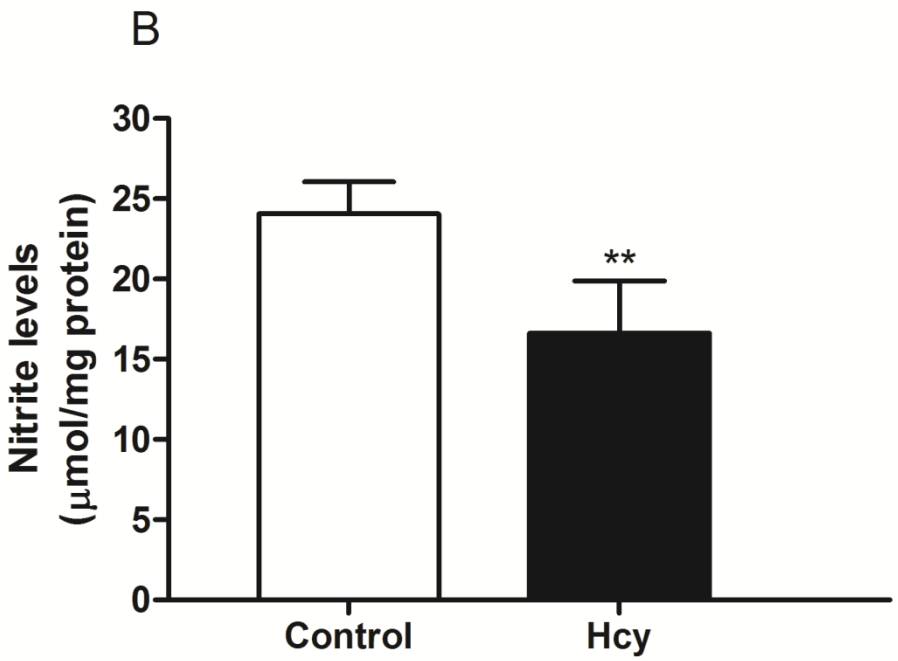
B
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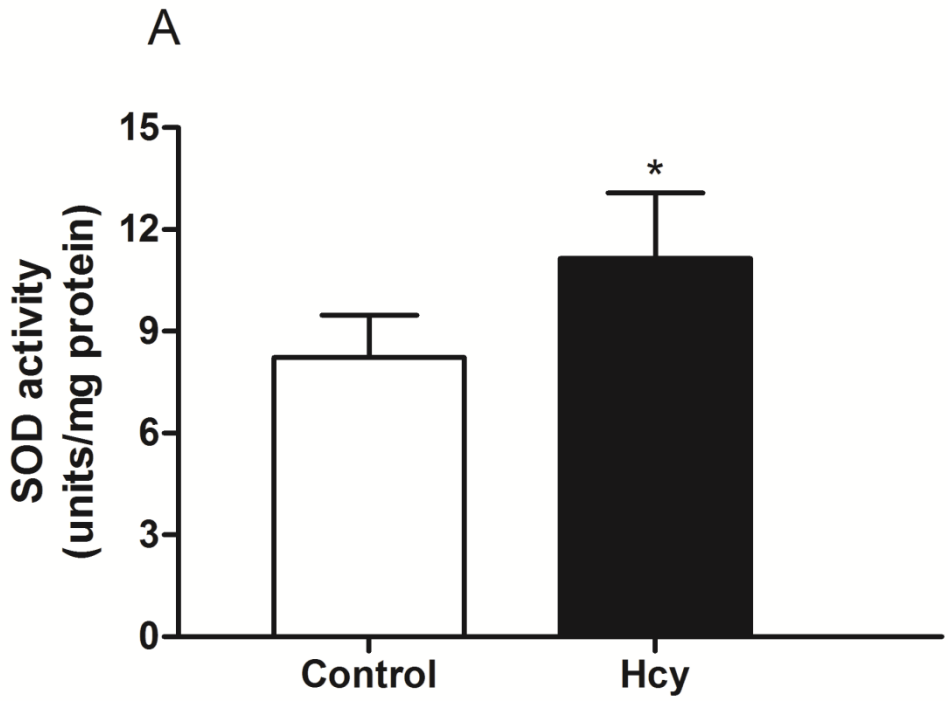
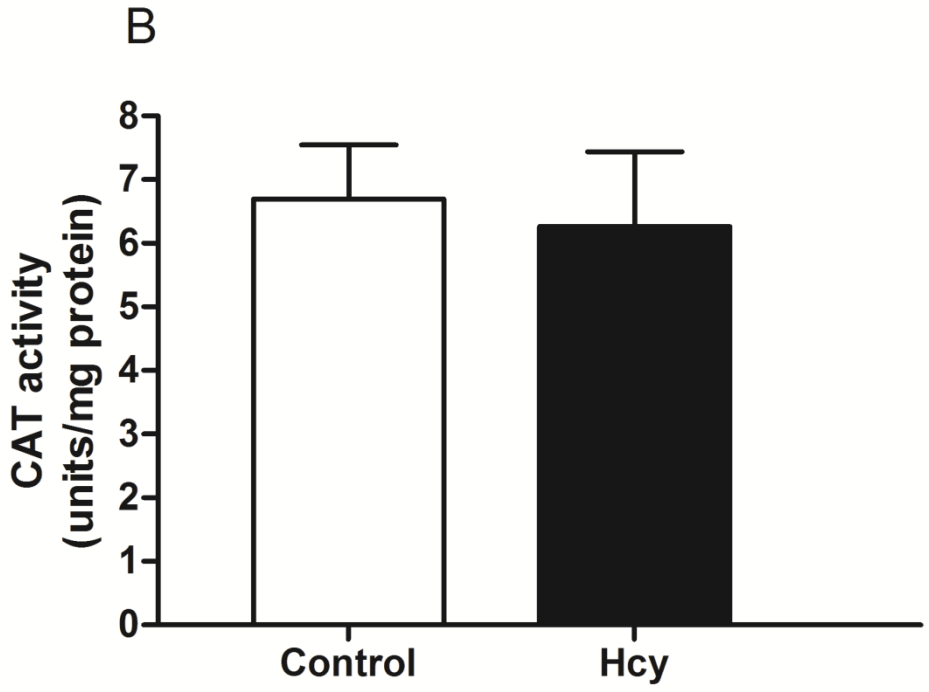
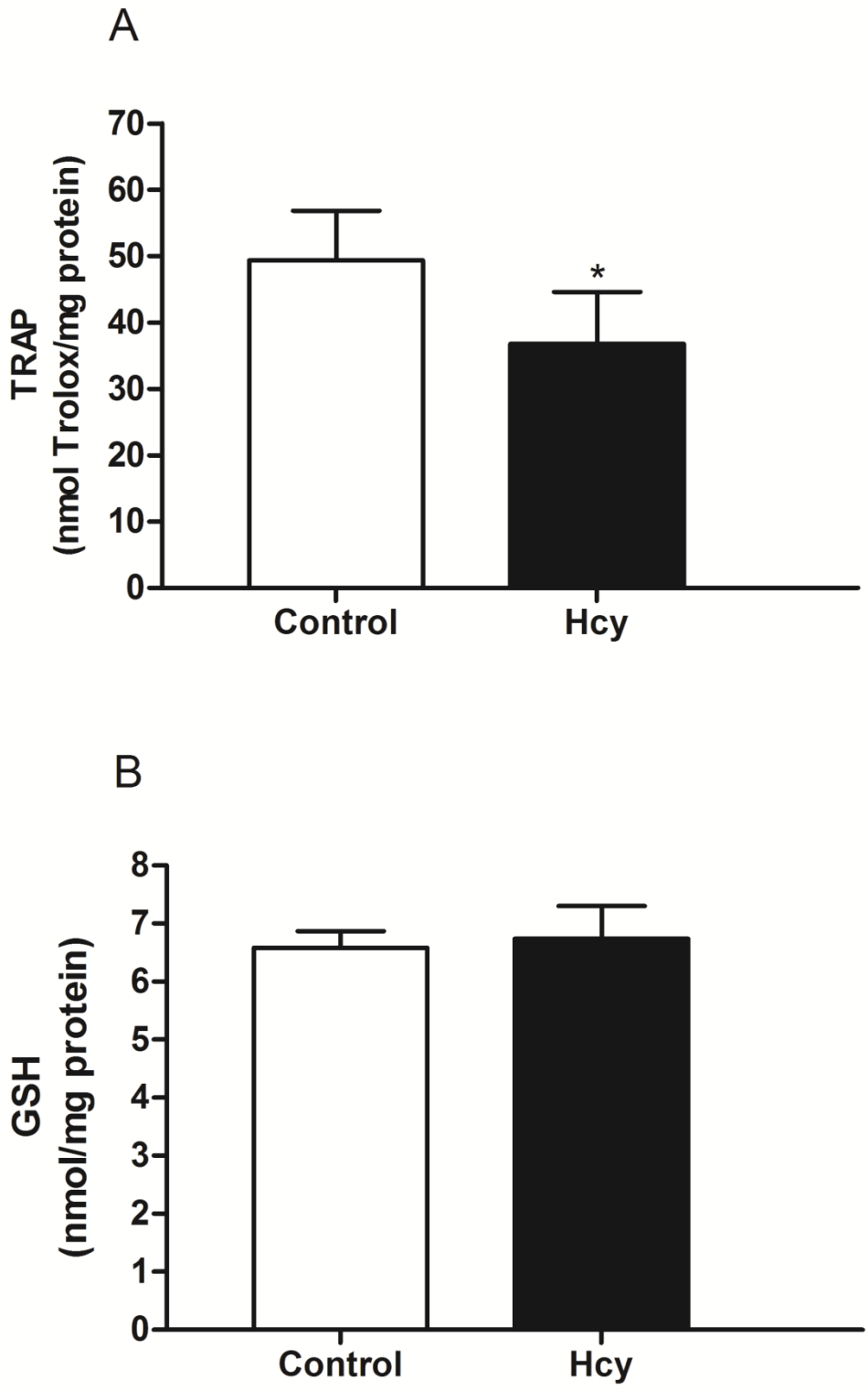
25
20
15
10
5
0
**
Control
Hcy


A
SOD activity
(units/mg protein)
15
12
9
6
3
0
*
Control
Hcy


B
CAT activity
(units/mg protein)
8
7
6
5
4
3
2
1
0
Control
Hcy


A
TRAP
(nmol Trolox/mg protein)
70
60
50
40
30
20
10
0
*
Control
Hcy
B
GSH
(nmol/mg protein)
8
7
6
5
4
3
2
1
0
Control
Hcy

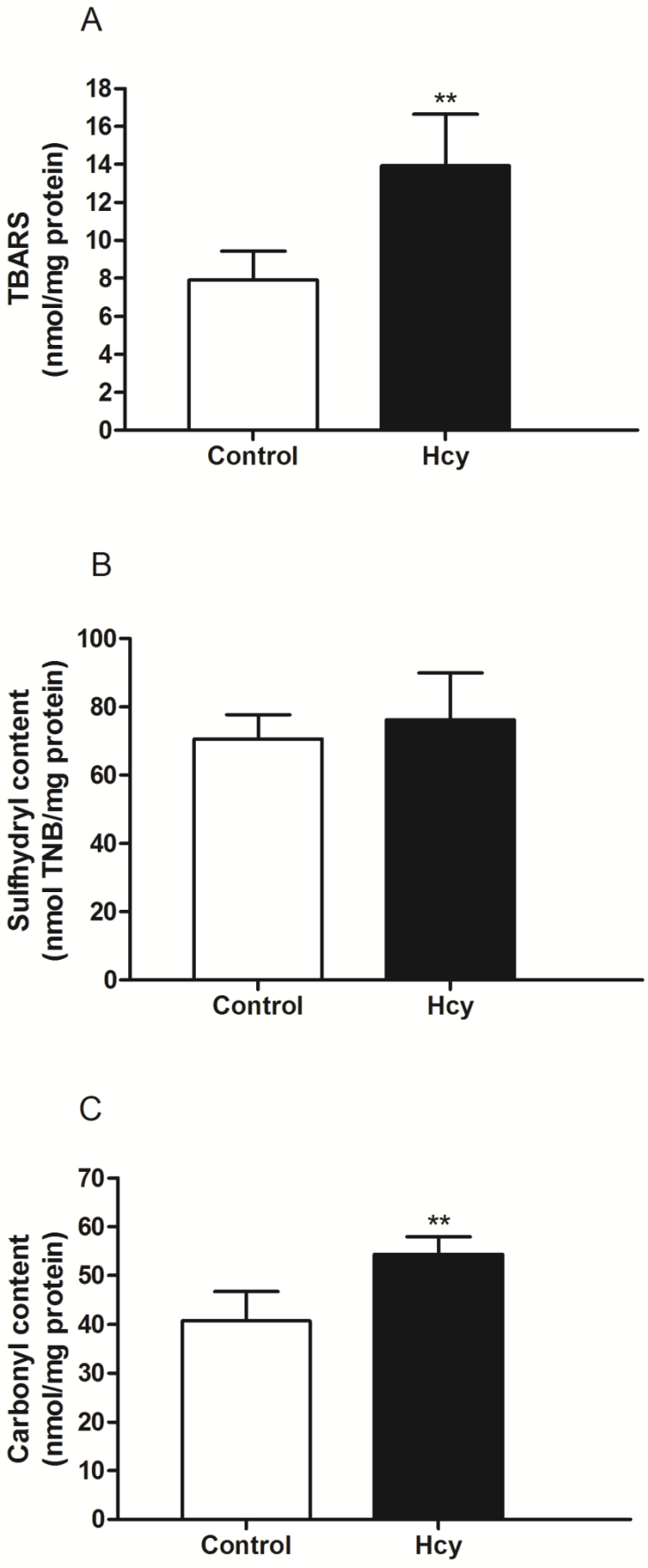

A
TBARS
(nmol/mg protein)
18
16
14
12
10
8
6
4
2
0
**
Control
Hcy
B
Sulfhydryl content
(nmol TNB/mg protein)
100
80
60
40
20
0
Control
Hcy
C
Carbonyl content
(nmol/mg protein)
70
60
50
40
30
20
10
0
**
Control
Hcy

Table I. Homocysteine levels in the cardiac tissue after chronic administration

Group	Homocysteine levels (nmol/g tissue)
Saline	0.88 ± 0.05
Hcy - 30 minutes	5.30 ± 0.98
Hcy - 12 hours	1.81 ± 0.70

Note: Hcy was administered from the 30th to the 60th day of life. Control rats received saline solution in the same volumes. Data are expressed as mean ± S.D. for 5 rats in each group.

Table II. Parameters of oxidative stress in heart of hyperhomocysteinemic rats

Parameters	Control	Homocysteine
DCF *(nmol/mg protein)*	139.88 ± 15.82	153.32 ± 5.53
Nitrite levels *(µmol/mg protein)*	7.63 ± 1.02	8.64 ± 1.19
SOD activity *(units/mg protein)*	6.74 ± 1.23	6.97 ± 0.82
CAT activity *(units/mg protein)*	6.41 ± 0.75	6.53 ± 0.22
TRAP *(nmol Trolox/mg protein)*	86.90 ± 12.54	69.34 ± 7.43*
GSH content *(nmol/mg protein)*	1.82 ± 0.13	1.86 ± 0.13
TBARS levels *(nmol/mg protein)*	0.95 ± 0.1	0.93 ± 0.083
Sulfhydryl content *(nmolTNB/mg protein)*	29.51 ± 2.63	29.01 ± 5.77
Carbonyl content *(nmol/mg protein)*	4.40 ± 0.41	6.51 ± 1.11**

Note: * significantly different from control ($p< 0.05$)
** significantly different from control ($p< 0.01$)

Capítulo III

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Mild hyperhomocysteinemia reduces the activity and immunocontent, but does not alter the gene expression, of catalytic α subunits of cerebral Na^+,K^+-ATPase

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Abstract Na^+,K^+-ATPase is a membrane protein which plays a key role in the maintenance of ion homeostasis that is necessary to neuronal excitability, secondary transport and neurotransmitter uptake. Mild hyperhomocysteinemia leads to several clinical manifestations and particularly cerebral diseases; however, little is known about the mechanisms of homocysteine on cerebral Na^+,K^+-ATPase. In the present study, we investigated the effect of mild hyperhomocysteinemia on the activity, the immunocontent of catalytic subunits (α_1, α_2, and α_3) and the gene expression of this enzyme. We used the experimental model of mild hyperhomocysteinemia that was induced by homocysteine administration (0.03 μmol/g of body weight) twice a day, from the 30th to the 60th postpartum day. Controls received saline in the same volumes. Results showed that mild hyperhomocysteinemia significantly decreased the activity and the immunocontent of the α_1 and α_2 subunits of the Na^+,K^+-ATPase in cerebral cortex and hippocampus of adult rats. On the other hand, we did not observe any change in levels of Na^+,K^+-ATPase mRNA transcripts in such cerebral structures of rats after chronic exposure to homocysteine. The present findings support that the homocysteine modulates the Na^+,K^+-ATPase and this could be associated, at least in part, with the risk to the development of cerebral diseases in individuals with mild hyperhomocysteinemia.

Keywords Mild hyperhomocysteinemia · Na^+,K^+-ATPase · Catalytic subunits · Gene expression · Cerebral structures

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Introduction

Na^+,K^+-ATPase, or Na^+, K^+ pump, is an integral membrane protein that regulates neuronal signaling, ion homeostasis, muscle contraction, and substrate transportation of most animal cells [1]. This enzyme transports 3 Na^+ from within the cell in exchange for 2 K^+ from outside the cell using the energy derived from hydrolysis of one molecule of ATP [2]. Structurally, Na^+,K^+-ATPase consists of three subunits, α (110 kDa), β (31 kDa), and γ (7–17 kDa) [2, 3]. The α subunit is responsible for the catalytic activity of the enzyme and contains the binding sites for Na^+, K^+, ATP, and allosteric sites for inhibitors and activators [4, 5]. The role of the β subunit seems to be associated with the facilitation of the insertion of α subunit into the plasma membrane. The γ subunit apparently modulates the activity of Na^+,K^+-ATPase [6].

It has been identified four isoforms of the α subunit (α_1, α_2, α_3, and α_4) and three isoforms of the β subunit (β_1, β_2, and β_3) of the Na^+,K^+-ATPase: α_1 and β_1 are ubiquitously expressed; α_2 is expressed mainly in skeletal muscle and brain (astrocytes), α_3 is expressed by neurons and heart cells, while α_4 is expressed only in testes [1, 2, 7]; β_2 is expressed by neurons and muscle and β_3 by lung, liver, and testes [8, 9]. The different associations between the Na^+,K^+-ATPase subunits contribute to the formation

of multiple isoforms of the enzyme in tissues and cells [1, 10, 11].

Disturbance in Na^+,K^+-ATPase may induce significant damage on brain function. The inhibition of its activity is found in various diseases that affect the central nervous system (CNS), including schizophrenia [12], cerebral ischemia [13], Alzheimer's disease [14], and animal models of inborn errors of metabolism [15–18], depression [19] and mania [20].

Mild hyperhomocysteinemia, characterized by an elevated plasma homocysteine [Hcy] concentration between 15 and 30 μmol/L [21], is a common condition in many populations since it can be caused by vitamin deficiencies, such as vitamin B_6, vitamin B_{12}, and folate, impaired renal function, smoking, alcohol consumption, stress, drug treatment, and others [22–25]. Several studies support an association between mild hyperhomocysteinemia and cerebral diseases, but the mechanisms mediating Hcy-induced cerebral alterations are not completely defined.

Considering that the Na^+,K^+-ATPase plays an crucial role in maintenance of cerebral homeostasis and that alterations in its function can lead to several disorders in CNS, the objective of this study was to investigate the effect of mild hyperhomocysteinemia on activity, immunocontent, and gene expression of catalytic subunits (α_1, α_2 and α_3) of the Na^+,K^+-ATPase in cerebral cortex and hippocampus of adult rats.

Experimental procedure

Animals and reagents

Wistar rats (30 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animals were divided into two experimental groups: control treated with saline 0.9 % ($n = 18$) and Hcy-treated ($n = 18$) and were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20 % (w/w) protein commercial chow. Animal care followed the ''Principles of Laboratory Animal Care'' (NIH publication 85-23, revised 1996) and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (#19634).

Chronic mild hyperhomocysteinemia

DL-Hcy (0.03 μmol/g of body weight) was administered subcutaneously twice a day from the 30th to the 60th day of life of rats; control rats received saline solution in the same volumes (0.5 ml/100 g of body weight). Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those described in the plasma of patients with mild hyperhomocysteinemia (30 μM) [26]. The animals were killed by decapitation 12 h after the last injection of Hcy and the cerebral cortex and hippocampus were removed.

Tissue preparation

The cerebral cortex and hippocampus were homogenized in 10 volumes (1:10, w/v) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. The homogenates were centrifuged at 1,000×*g* for 10 min; the supernatants were removed for Na^+,K^+-ATPase activity determination.

Determination of Na^+,K^+-ATPase activity

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μl. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of ATP to a final concentration of 3.0 mM and was incubated for 20 min. Controls were carried out under the same conditions with addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays [13]. Released inorganic phosphate (Pi) was measured by the method of [27] and enzyme specific activity was expressed as nmol Pi released per min per mg of protein.

Western blot analysis

Cerebral cortex and hippocampus of rats were homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4 % SDS. For electrophoresis analysis, samples were dissolved in 25 % (v/v) of a solution containing 40 % glycerol, 5 % mercaptoethanol, and 50 mM Tris–HCl, pH 6.8. Equal protein concentrations were loaded onto 7.5 % polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). The gels were transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20 % methanol, and 0.25 % SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5 % bovine serum albumin, fraction V). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05 % Tween-20 (T-TBS) and then incubated overnight at 4 °C in blocking solution containing one of the following

monoclonal antibodies: anti-b actin diluted 1:500, monoclonal anti-Na^+,K^+-ATPase (α_1 subunit) antibody clone M8-P1-A3 diluted 1:1,000 obtained from Sigma (St. Louis, MO, USA), Na^+,K^+-ATPase α_2-isoform diluted 1:1,000 from Millipore (Millipore, Billerica, MA, USA) and monoclonal anti-Na^+,K^+-ATPase (α_3 subunit) antibody clone XVIF9-G10 obtained from Sigma (St. Louis, MO, USA). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario).

Analysis of gene expression by semi-quantitative RT–PCR

The analysis of Na^+,K^+-ATPase catalytic subunits expression were carried out by semi-quantitative reverse transcriptase polymerase chain reaction (RT–PCR) assay. The cerebral cortex and hippocampus were dissected and immediately frozen in liquid nitrogen for storage in −80 °C freezer. The total RNA extraction was performed using TRIZOL® reagent (Invitrogen) in accordance with the manufacturer instructions. The cDNA species were synthesized with the ImPRO-II Reverse Transcriptase for RT–PCR from 1 µg of total RNA and oligo (dT) primer following the suppliers' instructions. cDNA (1 µL) was used as a template for PCR with the specific primers for Na^+,K^+-ATPase catalytic subunits (Table 1). β-actin–PCR was carried out as an internal standard. PCR reactions were performed with a total volume of 25 µL using a final concentration of 0.08 µM of each primer indicated below, 1.6 mM of $MgCl_2$, and 0.5 U Taq Platinum Polymerase in the supplied reaction buffer. Conditions for Na^+,K^+-ATPase catalytic subunits PCR were as follows: initial 2 min denaturation step at 94 °C; 1 min at 94 °C, 1 min annealing step at 62 °C, 1 min extension step at 72 °C for 30 cycles, and a final 10 min extension at 72 °C. Conditions for β-actin PCR were as follows: initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step at 58.5 °C, 1 min extension step at 72 °C for 35 cycles, and a final 10 min extension at 72 °C. PCR products were analyzed on an electrophoresis 1 % agarose gel containing GelRed® (Biotium). The relative abundance of each mRNA versus β-actin (enzyme/β-actin) was determined by densitometry using the freeware ImageJ 1.37 for Windows.

Protein determination

Protein was measured by the Coomassie Blue method according to [28] using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by Student's t test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

Results

Initially, we investigated the influence of Hcy on Na^+,K^+-ATPase specific activity in cerebral cortex and hippocampus of rats submitted to chronic mild hyperhomocysteinemia (Fig. 1). Statistical analyses revealed that Hcy significantly inhibited Na^+,K^+-ATPase specific activity in cerebral cortex [$t(10) = 7.053$; $p < 0.001$], and hippocampus [$t(10) = 5.151$; $p < 0.001$] of adults rats when compared to control.

In order to investigate whether the decrease in Na^+,K^+-ATPase activity caused by mild hyperhomocysteinemia was due to a reduction in the levels of enzyme molecules, we measured the immunocontent of the catalytic α_1, α_2, and α_3 subunits of the Na^+,K^+-ATPase in cerebral

Table 1 PCR primers sequences of Na^+,K^+-ATPase catalytic subunits and β-actin

Gene		Sequence (5′–3′)	PCR product (bp)	Annealing temperature (°C)	Cycles
Alpha1	Sense	TCTATGGACGACCATAAACTCAGCCTGG	297	62	30
	Antisense	AGCAGACAGCACGACCCCGAGGTAC			
Alpha2	Sense	ACCAAGTGGATCTGTCCAAGGGCCTC	292	62	30
	Antisense	GCTTCCTGGTAGTAGGAGAAGCAGCCAG			
Alpha3	Sense	AAAGATGACAAGAGCTCGCCCAAGAAG	538	62	30
	Antisense	TGATCTCCACCAGGTCCCCGACCAC			
Actb	Sense	TATGCCAACACAGTGCTGCTGG	210	58.5	35
	Antisense	TACTCCTGCTTCCTGATCCACAT			

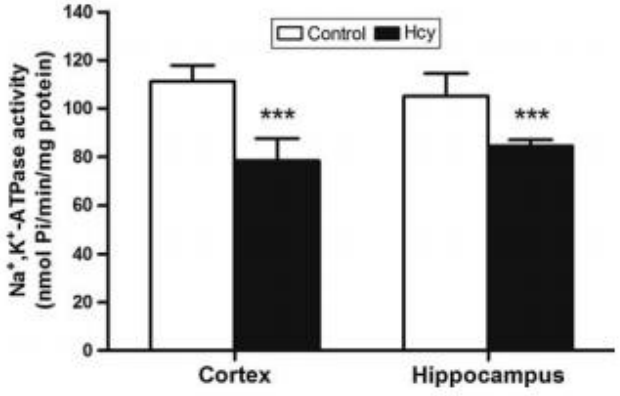

Fig. 1 Effect of chronic homocysteine administration on Na^+,K^+-ATPase specific activity in homogenates from cerebral cortex and hippocampus of adult rats. Results are expressed as mean ± SD for 6 animals in each group. Different from control, $***p < 0.001$ (Student's t test)

cortex and hippocampus of adult rats. As reported in Fig. 2, the mild hyperhomocysteinemia significantly decreased the immunocontent of the α_1 and α_2 subunits of the Na^+,K^+-ATPase in cerebral cortex [$t(8) = 6.165$; $p < 0.001$; $t(9) = 3.793$; $p < 0.01$], and hippocampus [$t(10) = 4.007$; $p < 0.01$; $t(10) = 3.662$; $p < 0.01$], respectively. On the other hand, the immunocontent of α_3 subunit was not altered by Hcy treatment.

Finally, we analyzed the relative expression of the Na^+,K^+-ATPase catalytic α_1, α_2, and α_3 subunits after chronic exposure to Hcy by semi-quantitative RT–PCR. As shown in Fig. 3, the relative expressions of the isoforms α_1, α_2, and α_3 of the Na^+,K^+-ATPase were not altered by mild hyperhomocysteinemia in cerebral cortex [$t(6) = 0.062$; $p > 0.05$; $t(6) = 1.300$; $p > 0.05$; $t(6) = 0.180$; $p > 0.05$] and hippocampus [$t(6) = 1.395$; $p > 0.05$; $t(6) = 0.083$; $p > 0.05$; $t(6) = 1.906$; $p > 0.05$], respectively.

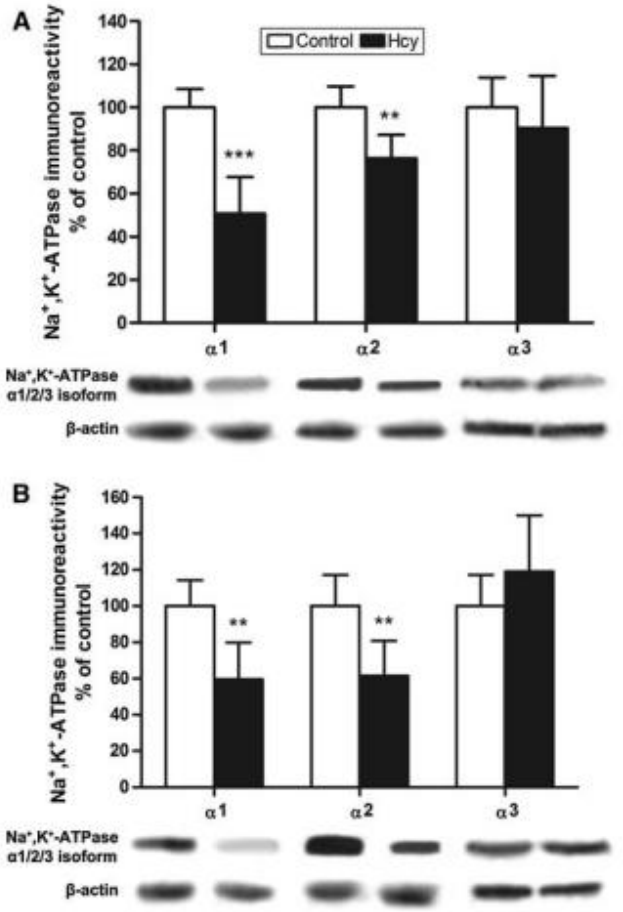

Fig. 2 Effect of chronic homocysteine administration on immunocontent of the α_1, α_2, and α_3 subunits of the Na^+,K^+-ATPase in homogenate of cerebral cortex (**a**) and hippocampus (**b**) of rats. All lanes received equivalent amounts (50 μg) of total protein from cell extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean ± SD for 4–7 animals in each group. Different from control, $**p < 0.01$; $***p < 0.001$ (Student's t test)

Discussion

Na^+,K^+-ATPase activity has been related to pathological conditions that affect the CNS because it controls the ionic environment essential for neuronal activity and neurotransmitter uptake [29], consuming about 40–50 % of the ATP generated in the brain [30]. On the other hand, the brain is vulnerable to high plasma Hcy levels because this amino acid is uptake by membrane transporter leading to intracellular accumulation [31]. Moreover, the brain lacks two major metabolic pathways for Hcy elimination: betaine remethylation and transsulfuration [32]. It has been reported that rats subjected to experimental severe hyperhomocysteinemia, where plasma Hcy levels (±500 μM) are similar to those found in human classical homocystinuria, present significant inhibition of Na^+,K^+-ATPase activity in hippocampus, parietal, prefrontal, and cingulate cortices [16, 33].

Mild hyperhomocysteinemia (15–30 μM Hcy) has been considered a risk factor to cognitive disabilities and neurological disorders such as Alzheimer's disease, age-related dementias, and Parkinson's disease, whose mechanisms have been extensively investigated [34–38]. However, up to now, there is no evidence of effect of mild hyperhomocysteinemia on Na^+,K^+-ATPase. In this work, we initially demonstrated that chronic mild hyperhomocysteinemia significantly reduces Na^+,K^+-ATPase activity in cerebral cortex and hippocampus of adult rats.

It is well described that Na^+,K^+-ATPase activity is highly vulnerable to oxidative insult through of disruption of phospholipid microenvironment of the enzyme, since

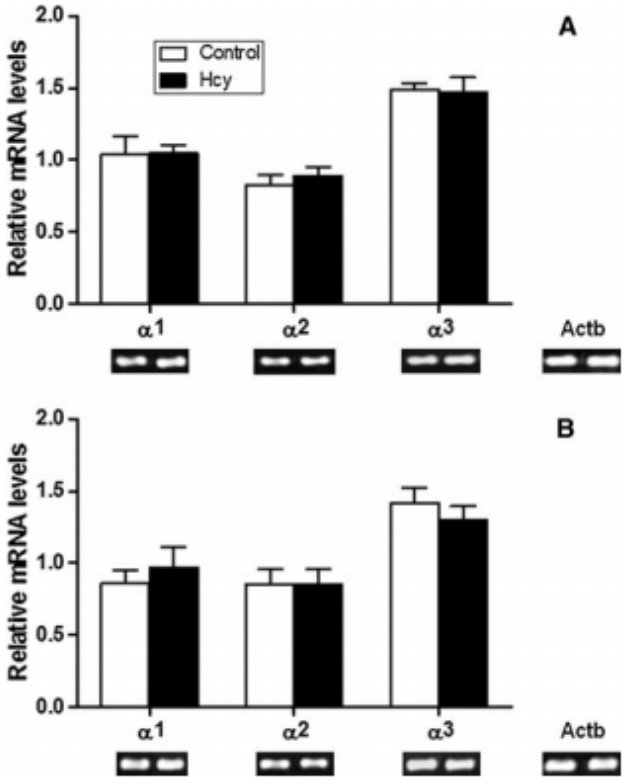

Fig. 3 Gene expression of α_1, α_2, and α_3 subunits of the Na^+,K^+-ATPase and β-actin after homocysteine administration in cerebral cortex (**a**) and hippocampus (**b**) of rats. Results are expressed as mean ± SD for 4–5 animals in each group

Na^+,K^+-ATPase is a membrane-embedded protein, or direct damage to enzyme by reactive oxygen species and/or lipid peroxidation products [39–41]. Indeed, previous study from our group showed that rats subjected to mild hyperhomocysteinemia presented increase in reactive oxygen species and thiobarbituric acid reactive substances [TBARS] levels, an index of lipid peroxidation, in cerebral cortex [26]. Therefore, it is plausible to suggest that the oxidative damage promoted by Hcy may alter the activity of this enzyme.

Differences in expression profiles of the α subunits of the Na^+,K^+-ATPase together with specific characteristics in affinities for Na^+, K^+, cardiotonic steroids, and in voltage sensitivity, indicate varied physiological roles of the different isoforms [42, 43]. The α_1, α_2, and α_3 isoforms are found in different cell types of the brain; in contrast to the α_4 isoform that is not CNS-related. Neurons are the principal sources of the α_3, whereas glial cells preferentially express α_2 subunit [44, 45]. Considering that the chronic mild hyperhomocysteinemia inhibits Na^+,K^+-ATPase activity, we extend our study and investigated whether the Hcy could alter the immunocontent and gene expression of the catalytic subunits of the enzyme in the cerebral cortex and hippocampus of adult rats. Results showed that the immunocontent of α_1 and α_2 isoforms were significantly decreased, while α_3 was not altered in these cerebral structures. On the other hand, the gene expression pattern of the catalytic subunits of the Na^+,K^+-ATPase was not affected by mild hyperhomocysteinemia, suggesting that other factors could affect the relation between mRNA and protein expression, such as protein degradation rate and posttranscriptional mRNA modifications.

Na^+,K^+-ATPase is modulated by different mechanisms activated in response to changing cellular requirements [1]. There is evidence that isoform-specific regulation of activity may occur under the influence of neurotransmitters [46, 47], and through regulatory phosphorylation [48]. Studies show that the amount of enzyme at the plasma membrane can be modified by alterations in the rate of synthesis or degradation of the individual polypeptides, as well as by mobilization of enzyme molecules from the endosomal pools to the cell surface [1, 49]. In addition, Lima et al. [50] demonstrated a significant correlation negative between the decrease in Na^+,K^+-ATPase α_1 subunit with the increase in TBARS and protein carbonyl content after traumatic brain injury. In this context, we suggest that the oxidative stress promoted by mild hyperhomocysteinemia could lead to conformational changes on this enzyme, altering the immunoreactivity of α_1 and α_2 Na^+,K^+-ATPase subunits. The reduction in the content of catalytic subunits could decrease the levels of available enzyme molecules and consequently their activity.

Given its important role, there is evidence suggesting that Na^+,K^+-ATPase activity alterations may be associated with neurotoxic mechanisms [51, 52]. Pump inhibition causes an increase in the cellular Na^+ concentration, which can lead to changes in intracellular pH (via the Na:H exchange system), or intracellular Ca^{+2} concentration through the Na:Ca exchanger [2]. These alterations can have profound effects on neuronal excitability, secondary transport, and neurotransmitter signaling in brain. Na^+,K^+-ATPase dysfunction has also been associated with the impairment in spatial learning impairment and anxiety-related behavior [53]. Furthermore, clinical studies have linked changes in the enzyme activity with depressive [54] and mood disorders [55].

It has been reported that in astrocytes the α_2 isoform of the Na^+,K^+-ATPase is co-localized with different glutamate transporters forming a protein-complex. These transporters are sodium dependent proteins, using electrochemical sodium gradient generated by pump activity to drive the uphill transport of glutamate [56]. On the other hand, the glutamatergic excitotoxicity appears to be associated with brain damage caused by Hcy [57]. In vitro experiments have shown that the exposure of cultured cortical and hippocampal neurons to Hcy increases their vulnerability to excitotoxicity [58, 59]. Thus, we believe that the decrease in α_2 subunit immunocontent might be

associated with the glutamatergic excitotoxicity caused by Hcy, resulting in accumulation of glutamate in the synaptic cleft. Consistent with these findings, we previously showed that severe hyperhomocysteinemia reduces glutamate uptake in parietal cortex [60] and hippocampus of rats [61].

To our knowledge, this study was the first demonstration that experimental mild hyperhomocysteinemia inhibits Na^+,K^+-ATPase and reduces immunocontent without altering the gene expression of catalytic subunits of this enzyme in cerebral cortex and hippocampus of adult rats. Based on these, it is plausible to suggest that the association of Hcy with cerebral diseases may be related, at least in part, to injury on Na^+,K^+-ATPase.

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Capítulo IV

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Chronic mild hyperhomocysteinemia alters ectonucleotidase activities and gene expression of ecto-5′-nucleotidase/CD73 in rat lymphocytes

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Abstract Since mild hyperhomocysteinemia is a risk factor for cardiovascular and cerebral diseases and extracellular nucleotides/nucleosides, which are controlled by the enzymatic action of ectonucleotidases, can induce an immune response, in the present study, we investigated the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities and expression in lymphocytes from mesenteric lymph nodes and serum of adult rats. For the chronic chemically induced mild hyperhomocysteinemia, Hcy (0.03 μmol/g of body weight) or saline (control) were administered subcutaneously from the 30th to the 60th day of life. Results showed that homocysteine significantly decreased ATP, ADP, and AMP hydrolysis in lymphocytes of adult rats. E-NTPDases transcriptions were not affected, while the ecto-5′-nucleotidase transcription was significantly decreased in mesenteric lymph nodes of hyperhomocysteinemic rats. ATP, ADP, and AMP hydrolysis were not affected by homocysteine in rat serum. Our findings suggest that Hcy in levels similar to considered risk factor to development of vascular diseases modulates the ectonucleotidases, which could lead to a pro-inflammatory status.

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Introduction

Homocysteine (Hcy) is a thiol amino acid synthesized during the metabolic conversion of methionine to cysteine [1]. Hyperhomocysteinemia occurs when the plasma concentration of Hcy exceeds 15 μmol/l, being classified into three ranges; mild (15–30 μmol/l), moderate (31–100 μmol/l), and severe (>100 μmol/l) [2]. Mild hyperhomocysteinemia is prevalent in the general population and can be caused mainly by nutritional deficiencies of folate or vitamin B_{12}, renal disease, and certain medications [3–5]. Some studies support an association between mild hyperhomocysteinemia and vascular diseases [6–8]. The mechanisms mediating Hcy-induced vascular changes are not completely defined, but it is established that hyperhomocysteinemia leads to endothelial dysfunction by promoting oxidative stress and inflammation [9].

Since the strategy of using animal models is useful to better understand the pathophysiology of diseases, we have developed an experimental model of chronic mild hyperhomocysteinemia in adult rats [10], whose plasma levels of Hcy were similar to those considered as risk factor for cardiovascular and cerebral diseases [11, 12]. Animals subjected to this experimental model present oxidative damage in blood and cerebral cortex [10].

Extracellular tri- and diphosphate nucleotides, such as adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP), together with adenosine (Ado), modulate a multiplicity of tissue functions, including development, blood flow, inflammation, and immune reactions [13]. These molecules exert their effects by binding to two types

of purinoceptors: P1 and P2. There are four subtypes of P1 receptors: A_1, A_{2A}, A_{2B}, and A_3, which respond to the nucleoside Ado. P2 receptors are activated by ATP and ADP and classified into two families: ionotropic P2X, subdivided in seven subtypes ($P2X_{1-7}$), and G-protein-coupled P2Y, characterized by eight subtypes ($P2Y_{1,2,4,6,11-14}$) [14]. It has been shown that ATP exerts a pro-inflammatory role and induces cytokine release by acting at $P2X_7$ receptors, while the Ado has anti-inflammatory actions via binding to A_{2A} receptors, found in several cells, such as neutrophils, monocytes/macrophages, lymphocytes, platelets, and neurons [15, 16].

The levels of nucleotides in the extracellular space are controlled by the enzymatic action of ectonucleotidases. Ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) are a family of ecto-enzymes that hydrolyze extracellular ATP and ADP to AMP, and this nucleotide monophosphate is converted to Ado by ecto-5′-nucleotidase, also identified as CD73. Four members of the NTPDase family (NTPDase1, 2, 3, and 8) are typical cell surface-located and have an active site facing to the extracellular milieu [13, 14, 17].

Considering that (a) hyperhomocysteinemia induces lymphocytes proliferation and differentiation [18, 19], (b) ATP, ADP, and Ado are signaling molecules that might play a role in the regulation of lymphocytes function [14], (c) Hcy added to the medium assay (in vitro studies) inhibits nucleotide hydrolysis in rat serum [20], and (d) to our knowledge there are no studies showing the in vivo effect of mild hyperhomocysteinemia on nucleotide hydrolysis, in the present study, we investigated the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in lymphocytes and serum of adult rats. We also evaluated the E-NTPDases and ecto-5′-nucleotidase expression in mesenteric lymph nodes, to better understand the involvement of extracellular nucleotide hydrolysis in mild hyperhomocysteinemia.

Materials and methods

Animals and reagents

Wistar rats (30 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air-conditioned constant temperature (22 ± 1°C) colony room, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the ''Principles of Laboratory Animal Care'' (NIH publication 85-23, revised 1985) and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (#19634).

Chronic mild hyperhomocysteinemia

Hcy (0.03 μmol/g of body weight) was administered subcutaneously from the 30th to the 60th day of life. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those described in the plasma of patients with mild hyperhomocysteinemia (30 μM) [10]. Control rats received saline solution in the same volumes (0.5 ml/100 g of body weight). The animals were killed by decapitation 12 h after the last injection of Hcy, and the blood and mesenteric lymph nodes were removed.

Isolation of lymphocytes

Mesenteric lymph nodes were removed and passed through a mesh grid in saline 0.9% [21]. Cells were washed three times with saline, and centrifuged at 200 g for 10 min. Afterward, cells were centrifuged 2 times at 200×*g* for 10 min with the same buffer used in the enzyme assays, without divalent cations [22]. The cells were counted with Trypan Blue, and only the groups with more than 95% of viability were used for the experiments.

Isolation of blood serum fraction

Blood samples were drawn after decapitation of rats and were soon centrifuged in plastic tubes at 5000×*g* for 5 min at 20°C. The serum samples obtained were then stored on ice and immediately used in the experiments [23].

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

The reaction medium contained 2 mM $CaCl_2$ (for ATP and ADP) or $MgCl_2$ (for AMP), 120 mM NaCl, 5 mM KCl, 60 mM glucose, 1 mM sodium azide, 0.1% mM albumin, and 20 mM Hepes buffer, pH 7.6, in a final volume of 200 μl. About 10^6 cells of lymphocytes were added to the reaction medium, and the enzyme reaction was started by the addition of ATP, ADP, or AMP to a final concentration of 2 mM and incubated for 30 min at 37°C. The reaction was stopped by the addition of 200 μl of 10% trichloroacetic acid (TCA). The samples were chilled on ice, and the amount of inorganic phosphate (Pi) released was measured as described by Chan et al. [24]. In order to correct nonenzymatic hydrolysis, we performed controls by adding the cells after reaction was stopped with TCA. All samples were assayed in triplicate. Enzyme activities were

generally expressed as nmol Pi released per min per 10^6 cells [22].

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

ATP, ADP, and AMP hydrolysis were determined using a method described by Yegutkin [25] with modification prescribed by Oses et al. [23]. The reaction mixture containing 3 mM ATP, ADP, or AMP as substrate, and 112.5 mM Tris–HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37°C for 40 min in a final volume of 200 µl. The reaction was stopped by the addition of 200 µl of 10% TCA. The samples were chilled on ice, and the amount of Pi released was measured as described by Chan et al. [24]. 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 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. All the samples were assayed in triplicate. Specific activities were generally expressed as nmol Pi released per min per milligram of protein.

Analysis of gene expression by semi-quantitative RT-PCR

Analysis of NTPDase1 (Entpd1), 2 (Entpd2), 3 (Entpd3), and ecto-5′-nucleotidase (Nt5e) gene expression was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. 12 h after the last injection of Hcy, mesenteric lymph nodes of rats were isolated for total RNA extraction with the Trizol® Reagent (Invitrogen) in accordance with the manufacturer's instructions. RNA purity was quantified spectrophotometrically and assessed by electrophoresis in a 1.0% agarose gel using GelRed™. The cDNA species were synthesized with SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) from 3 µg of total RNA following suppliers. RT reactions were performed for 50 min at 42°C. cDNA (1 µl) was used as a template for PCR with specific primers for Entpd1, 2, 3, and Nt5e. β-actin (Actb) was used for normalization as a constitutive gene. PCR reactions have a volume of 25 µl using a concentration of 0.4 µM of each primer indicated below and 200 µM and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. Conditions for all PCR were as follows: Initial 1 min denaturation step at 94°C, 1 min at 94°C, 1 min annealing step (Entpd1, 3, and Nt5e: 65°C; Entpd2: 66°C; Actb: 58.5°C), 1 min extension step at 72°C for 35 cycles and a 10 min final extension at 72°C. The amplification products were Entpd1—543 bp; Entpd 2—331 bp; Entpd 3—267 bp; Nt5e—403 bp; Actb—210 bp. For each set of PCR reactions, negative control was included. Ten microliters of the PCR reaction mixture were analyzed on a 1% agarose gel using GelRed™ and photographed under UV light. The Low DNA Mass Ladder was used as a molecular marker and normalized employing Actb (β-actin) as a constitutive gene. The images of stained PCR products were analyzed by optical densitometry and semi-quantified (enzyme/Actb mRNA ratios) using the computer software Image J. Table 1 shows the set of primers used.

Protein determination

Protein was measured by the Comassie Blue method according to Bradford [26], using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by Student's *t* test. All analyses were performed using the statistical package for the social sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $P < 0.05$.

Table 1 PCR primers sequences

Enzyme		Sequence (5′–3′)
Entpd1	Sense	GAT CAT CAC TGG GCA GGA GGA AGG
Entpd1	Antisense	AAG ACA CCG TTG AAG GCA CAC TGG
Entpd2	Sense	GCT GGG TGG GCC GGT GGA TAC G
Entpd2	Antisense	ATT GAA GGC CCG GGG ACG CTG AC
Entpd3	Sense	CGG GAT CCT TGC TGT GCG TGG CAT TTC TT
Entpd3	Antisense	TCT AGA GGT GCT CTG GCA GGA ATC AGT
Nt5e	Sense	CCC GGG GGC CAC TAG CAC CTC A
Nt5e	Antisense	GCC TGG ACC ACG GGA ACC TT
Actb	Sense	TAT GCC AAC ACA GTG CTG TCT GG
Actb	Antisense	TAC TCC TGC TTC CTG ATC CAC AT

Results

Effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in lymphocytes of adult rats

We measured extracellular ATP, ADP, and AMP hydrolysis in lymphocytes obtained of mesenteric lymph nodes of rats subjected to chronic mild hyperhomocysteinemia. Figure 1 shows that Hcy significantly decreased the hydrolysis of ATP [$t(8) = 6.144$; $P < 0.001$], ADP [$t(9) = 4.743$; $P < 0.01$], and AMP [$t(9) = 4.128$; $P < 0.01$] as compared to control.

Effect of chronic mild hyperhomocysteinemia on E-NTPDases and ecto-5′-nucleotidase expression in mesenteric lymph nodes of adult rats

As reported in Fig. 2, Entpd1, 2, and 3 transcript levels were not affected by chronic mild hyperhomocysteinemia [$t(6) = -1.974$; $P > 0.05$; $t(6) = 1.851$; $P > 0.05$; $t(7) = -1.246$; $P > 0.05$, respectively]. However, Nt5e mRNA transcript [$t(6) = 4.057$; $P < 0.01$] was decreased in mesenteric lymph nodes of rats.

Effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in serum of adult rats

We investigated the effect of chronic mild hyperhomocysteinemia on nucleotide hydrolysis in serum of adult rats. As observed in Fig. 3, ATP [$t(10) = -1.851$; $P > 0.05$], ADP [$t(12) = 0.406$; $P > 0.05$] and AMP [$t(15) = 0.092$; $P > 0.05$] hydrolysis were not affected by Hcy administration.

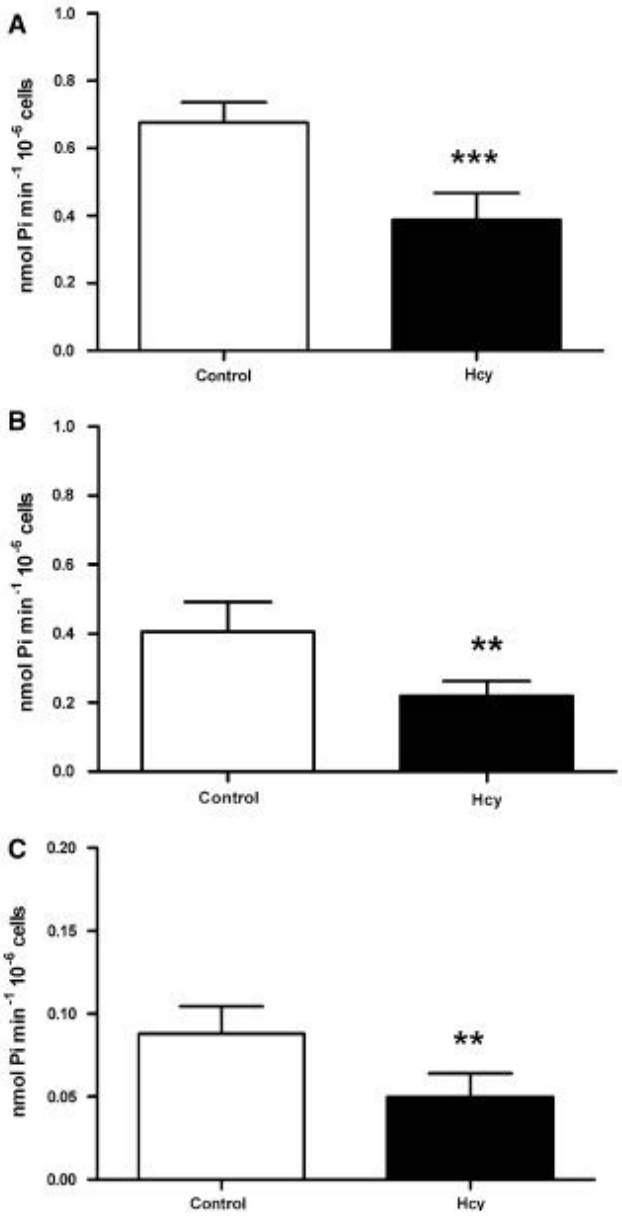

Fig. 1 Effect of chronic homocysteine administration on ATP (**a**), ADP (**b**), and AMP (**c**) hydrolysis in lymphocytes of adult rats. Results are expressed as mean ± SD for 4–6 animals in each group. Different from control, $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's t test). *Hcy* homocysteine

Discussion

Mild hyperhomocysteinemia is a risk factor for cerebral and cardiovascular diseases [12, 27, 28]. Although the mechanisms by which Hcy leads to these alterations are not well fully elucidated, there are studies showing that this amino acid may induce oxidative stress and inflammation, which may contribute to the development and progression of atherosclerotic plaques [9, 29–32].

ATP is present in high levels in the extracellular space during inflammation [33, 34]. This nucleotide may modulate inflammatory responses through several ways, including activating cells (endothelium, leukocytes), cytokine and chemokine release and increase adhesion molecules expression via activation of $P2X_7$ receptor [35–37]. ADP is the most important promoter of platelet aggregation, which may be secondary to vascular injury as a consequence of inflammatory processes [38]. On the other hand, nucleoside Ado exhibits potent anti-inflammatory and immunosuppressive action in tissues subjected to various forms of injurious stimuli such as ischemia and inflammation [39].

Since ectonucleotidases are important enzymes in the regulation of extracellular nucleotides and nucleosides levels, acting as signaling molecules in inflammatory processes, in the present study we evaluate the effect of chronic mild hyperhomocysteinemia on nucleotide hydrolysis in lymphocytes from mesenteric lymph nodes of adult

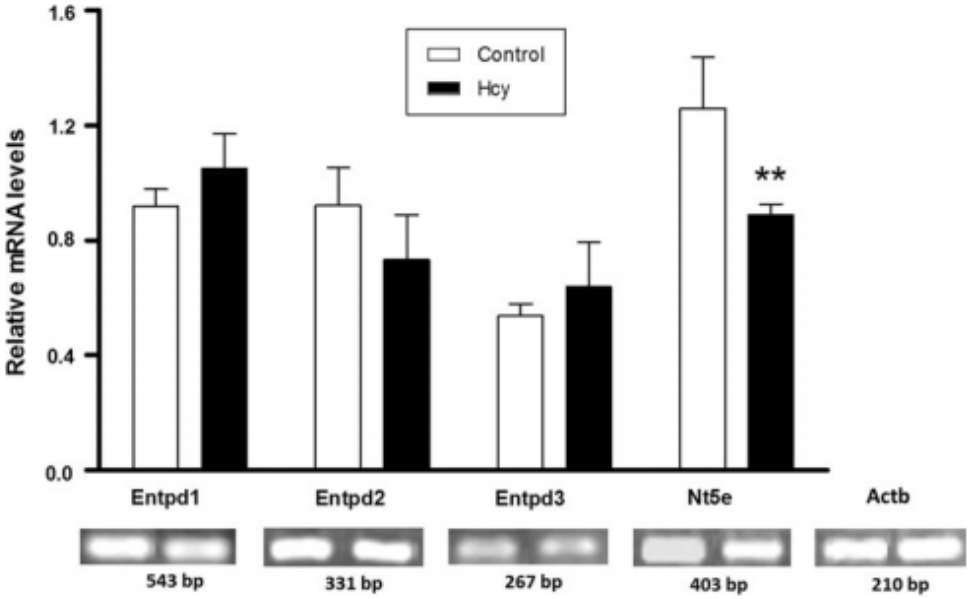

Fig. 2 Gene expression patterns after chronic Hcy treatment for NTPDase1 (Entpd1), NTPDase2 (Entpd2), NTPDase3 (Entpd3), ecto-5′-nucleotidase (Nt5e), and β-actin in mesenteric lymph nodes of rats. Results are expressed as mean ± SD for 4–5 animals in each group. Different from control, $^{**}P < 0.01$ (Student's *t* test). *Hcy* homocysteine

rats. Present reports show that extracellular ATP, ADP, and AMP hydrolysis were significantly decreased in lymphocytes from hyperhomocysteinemic rats.

In order to evaluate whether the mild hyperhomocysteinemia causes transcriptional modifications in the ecto-nucleotidases in lymphocytes, we also investigated the expression patterns of these enzymes after Hcy treatment in mesenteric lymph nodes of adult rats. A semi-quantitative RT-PCR analysis showed that E-NTPDase1, 2, and 3 transcriptions were not affected by chronic mild hyperhomocysteinemia. These findings demonstrated that the decrease in ATP and ADP hydrolysis caused by Hcy was not followed by alterations in E-NTPDases mRNA transcript levels. In this context, it well known that the gene expression is regulated by various factors involving cell machinery and signal transduction pathways, being that the enzymatic activity cannot be directly correlated with the gene expression pattern or with protein levels because of the existence of several post-translational events [40].

On the other hand, ecto-5′-nucleotidase/CD73 mRNA transcriptional level was significantly decreased in mesenteric lymph nodes of hyperhomocysteinemic rats. Considering that ATP and ADP can exert inhibitory effects on ecto-5′-nucleotidase/CD73 [41], it is possible that the inhibition in nucleotide hydrolysis caused by Hcy may lead to increase of ATP levels that consequently, inhibit the ecto-5′-nucleotidase/CD73. Taken together, these data could result in activation of inflammatory responses, since ATP could accumulate in the extracellular milieu, and AMP hydrolysis is decreased, leading to decreased Ado production.

Riksen et al. [42] showed that in patients with severe hyperhomocysteinemia, the cellular adenosine uptake is enhanced due to S-adenosyl-homocysteine (AdoHcy) formation, limiting Ado-induced vasodilation. However, to our knowledge, there are no studies showing the involvement of adenine nucleotides and nucleosides in mild hyperhomocysteinemia. In the present study, we suggest that the Hcy can reduce the Ado concentration through of inhibition of nucleotide hydrolysis in lymphocytes of rats. These findings corroborate with studies demonstrating that a decreased in Ado levels could be associated with the adverse effects of hyperhomocysteinemia, since several cardio- and vaso-protective actions are attributed to this nucleoside, such vasodilation [43], inhibition of platelet aggregation [44], and activation of cellular antioxidant enzyme systems [45].

Moreover, Ado regulates inflammatory actions on lymphocytes [36, 46]. The activation of the A_{2A} receptors on CD_4^+ T lymphocytes prevents myocardial ischemia–reperfusion injury by inhibiting the accumulation and activation of CD_4^+ T cells in the reperfused heart [47] and limits the production of inflammatory mediators such as IL-12, TNF-α, and INFγ by lymphocytes [48, 49]. In addition, Vuaden et al. [50] showed that a selective A_{2A} receptor agonist prevents the endotoxin-induced effects on nucleotide catabolism in mouse lymphocytes.

ATP and other nucleotides of adenine can be degraded by nucleotidases both in the membrane-bound form, and in the soluble form, located in the interstitial medium or within body fluids [51]. It is currently accepted that the exogenous ATP levels may be increased in several inflammatory and shock conditions, mainly as a consequence of nucleotide release from platelets, endothelial, and blood vessel cells [52–54]. This rise in exogenous ATP concentration is usually accompanied by concurrent secretion and/or cleavage of various enzymes to the extracellular milieu [55]. Therefore, serum enzymes might reduce the excess of the extracellular nucleotides levels and

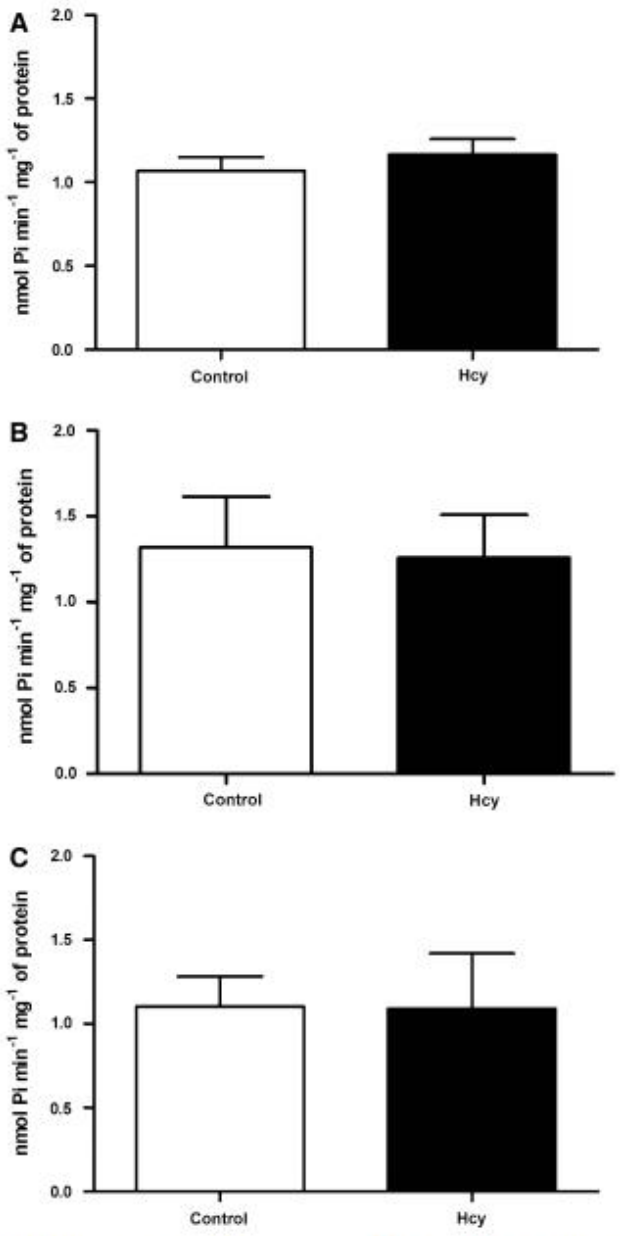

Fig. 3 Effect of chronic homocysteine administration on ATP (**a**), ADP (**b**), and AMP (**c**) hydrolysis in serum of adult rats. Results are expressed as mean ± SD for 6–9 animals in each group. *Hcy* homocysteine

to have an important role in maintaining normal physiology [23].

Our results also demonstrated that extracellular ATP, ADP, and AMP hydrolysis were not altered in serum of rats subjected to experimental mild hyperhomocysteinemia. The lack of effect of mild hyperhomocysteinemia on these parameters in serum is puzzling; however, Böhmer et al. [20] showed that the Hcy in vitro inhibits the nucleotide hydrolysis in the millimolar range, and that this inhibition is concentration dependent. It is important to consider that in vivo other factors could affect the enzymatic cascade and that our results represent concentrations of Hcy in the micromolar range, similar to those considered as a risk factor to cardio- and cerebrovascular diseases [11, 12].

In summary, in the present study, we demonstrated that experimental mild hyperhomocysteinemia significantly decreases the extracellular ATP, ADP, and AMP hydrolysis and ecto-5′-nucleotidase/CD73 gene expression pattern in lymphocytes of adult rats. Our findings suggest that the Hcy modulates the ectonucleotidases, probably decreasing the Ado levels, which could lead to a pro-inflammatory status.

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Capítulo V

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MILD HYPERHOMOCYSTEINEMIA ALTERS EXTRACELLULAR ADENINE METABOLISM IN RAT BRAIN

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Abstract—Since homocysteine (Hcy) is considered a risk factor to cerebral diseases and adenine nucleotides are important molecules to brain normal function, in the present study we investigated the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities and expression in rat cerebral cortex. The levels of ATP, ADP, AMP and adenosine (Ado) in cerebrospinal fluid (CSF) of adult rats also were evaluated by high-performance liquid chromatography. For the chronic chemically induced mild hyperhomocysteinemia, Hcy (0.03 μmol/g of body weight) was administered subcutaneously from the 30th to the 60th day of life. Control rats received saline solution in the same volumes. Results showed that Hcy significantly decreased nucleotide hydrolysis in the synaptosomal fraction and increased E-NTPDase1 and ecto-5′-nucleotidase transcripts in rat cerebral cortex. ATP levels were significantly increased, while Ado decreased in CSF of Hcy-treated rats. These findings suggest that the unbalance in ATP and Ado levels may be, at last in part, involved in the cerebral toxicity of mild hyperhomocysteinemia. © **2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: mild hyperhomocysteinemia, ectonucleotidases, ATP, adenosine, cerebral cortex, cerebrospinal fluid.

INTRODUCTION

Homocysteine (Hcy) is a sulfur-containing amino acid biosynthesized from methionine. In the first step of metabolic pathway, methionine is converted to S-adenosylmethionine (SAM), which serves as a methyl donor for methyl transferases. The major product of these transfer reactions is S-adenosylhomocysteine (SAH), which is rapidly hydrolyzed to Hcy and adenosine (Ado) in a reversible reaction catalyzed by SAH hydrolase (Williams and Schalinske, 2010).

Mild hyperhomocysteinemia, characterized by an elevated plasma Hcy concentration between 15 and 30 μmol/L (Raaf et al., 2011), is common in many populations whereas can be caused by thermolabile variant of methylenetetrahydrofolate reductase, vitamin deficiencies, drug treatment, aging, smoking, alcohol consumption, or can be secondary to systemic diseases such as insulin-dependent diabetes and hypothyroidism (Jacques et al., 2001; De Bree et al., 2002; Castro et al., 2006; Selhub, 2006; Troen et al., 2008). Furthermore, studies have shown that mild hyperhomocysteinemia is a risk factor to cardiovascular (De Bree et al., 2002; Huang et al., 2008) and neurodegenerative diseases (Obeid and Herrmann, 2006; Minagawa et al., 2010; Herrmann and Obeid, 2011).

ATP and Ado are signaling molecules that play crucial roles in the neurotransmission and neuromodulation acting by two types of purinoceptors: P1 receptors, activated by Ado, and P2 receptors that respond preferentially to ATP (Abbracchio et al., 2006). These molecules are metabolized by ectonucleotidases. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) is a family of ecto-enzymes composed of eight members responsible for hydrolyzing ATP and ADP to AMP that, by the action of an ecto-5′-nucleotidase, is converted to Ado (Yegutkin, 2008). Four members of this family (NTPDase1, 2, 3, and 8) are tightly bound to the plasma membrane via two transmembrane domains. Ecto-5′-nucleotidase is a homodimer linked to the plasma membrane through a glycosyl phosphatidylinositol lipid anchor. These enzymes have their active site facing the extracellular milieu (Robson et al., 2006; Yegutkin, 2008).

We previously developed an experimental model of chronic mild hyperhomocysteinemia in adult rats (Scherer et al., 2011) and showed that this model was able to induce a decrease in extracellular nucleotide hydrolysis and ecto-5′-nucleotidase/CD73 gene expression pattern in rat lymphocytes (Scherer et al.,

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Abbreviations: Actb, β-actin; Ado, adenosine; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid; E-NTPDases, Ecto-nucleoside triphosphate diphosphohydrolases; Hcy, homocysteine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; RT-PCR, reverse transcriptase-polymerase chain reaction; SAH, S-adenosylhomocysteine.

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2012). Therefore, in this study we sought to investigate the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities and gene expression in rat cerebral cortex. ATP, ADP, AMP and Ado concentrations in cerebrospinal fluid (CSF) of adult rats also were evaluated by high-performance liquid chromatography (HPLC).

EXPERIMENTAL PROCEDURES

Animals and reagents

Wistar rats (30 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were maintained on a 12:12-h light/dark cycle (lights on 07:00–19:00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the "Principles of Laboratory Animal Care"' (NIH publication 85-23, revised 1996) and was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil (#19634).

Chronic mild hyperhomocysteinemia

DL-Hcy (0.03 μmol/g of body weight) was administered subcutaneously twice a day from the 30th to the 60th day of life of rats; controls rats received saline solution in the same volumes (0.5 ml/100 g of body weight). Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those described in the plasma of patients with mild hyperhomocysteinemia (30 μM) (Scherer et al., 2011). The animals were killed by decapitation 12 h after the last injection of Hcy and the cerebral cortex and CSF were removed.

Subcellular fraction

The cerebral cortex was removed and placed in ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, pH 7.5, and 0.1 mM EDTA). The cerebral cortex was homogenized in five volumes of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as previously described (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000*g* for 20 min with the same ice-cold medium to remove the contaminating Percoll. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

Enzyme assays

The reaction medium used to assay the ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991) and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl. The synaptosome preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and the reaction was stopped by the addition of 200 μl 10% trichloroacetic acid. The reaction medium used to assay the AMP hydrolysis contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.0, and 0.15 M sucrose in a final volume of 200 μl (Heymann et al., 1984). The synaptosome preparation (10–20 μg protein) was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and was stopped by the addition of 200 μl 10% trichloroacetic acid. The released inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). In all enzyme assays, incubation times and protein concentration were chosen in order to ensure the linearity of the reactions (Heymann et al., 1984; Battastini et al., 1991). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in triplicate.

Analysis of gene expression by semi-quantitative RT-PCR

Analysis of NTPDase1 (Entpd1), 2 (Entpd2), 3 (Entpd3), and ecto-5′-nucleotidase (Nt5e) gene expression was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Twelve hours after the last injection of Hcy, cerebral cortex of rats was isolated for total RNA extraction with the Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The cDNA species were synthesized with Promega (Madison, Wisconsin, USA) from 3 μg of total RNA following suppliers. RT reactions were performed for 50 min at 42 °C. cDNA (1 μl) was used as a template for PCR with specific primers for Entpd1, 2, 3, and Nt5e. β-Actin (Actb) was used for normalization as a constitutive gene. PCR reactions have a volume of 25 μl using a concentration of 0.4 μM of each primer indicated below and 200 μM $MgCl_2$ and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. Conditions for all PCR were as follows: Initial 1-min denaturation step at 94 °C, 1 min at 94 °C, 1-min annealing step (Entpd1, 3, and Nt5e: 65 °C; Entpd2: 66 °C; Actb: 58.5 °C), 1-min extension step at 72 °C for 35 cycles and a 10-min final extension at 72 °C. The amplification products were: Entpd1 – 543 bp; Entpd2 – 331 bp; Entpd3 – 267 bp; Nt5e – 403 bp; Actb – 210 bp. For each set of PCR reactions, negative control was included. Five microliters of the PCR reaction mixture was analyzed on a 1% agarose gel using GelRed and photographed under UV light. The Low DNA Mass Ladder was used as a molecular marker and normalized employing Actb as a constitutive gene. The images of stained PCR products were analyzed by optical densitometry and semi-quantified (enzyme/Actb mRNA ratios) using the computer software Image J. Table 1 shows the set of primers used.

CSF purines measurement

Samples of CSF were centrifuged at 1 °C for 30 min at 16,000*g*, the supernatant taken and aliquots of 20 μl were applied to a reversed-phase HPLC system (Shimadzu, Kyoto, Japan) using a C_{18} column (Ultra C18, 25 cm × 4.6 mm × 5 μm, Restek, Bellefonte, PA, USA). Separation was carried out from 20 μl of CSF, with a reversed-phase column (Ultra C18, 25 cm × 4.6 mm × 5 μm, Restek) in a Shimadzu LC-10AD HPLC. The elution was carried out applying a linear gradient from 100% of solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6.0) to 100% of solvent B (solvent A plus 30% methanol) over a 30-min period (flow rate at 1.4 ml/min). The amounts of purines were measured by absorption at 260 nm. The retention time of standards was used as parameter for identification and quantification. Purines concentrations are expressed as nmol/ml.

Protein determination

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

Table 1. PCR primers sequences

Enzyme		Sequence (5′–3′)	
Entpd1	Sense	GAT CAT CAC TGG GCA GGA GGA AGG	543 bp
	Antisense	AAG ACA CCG TTG AAG GCA CAC TGG	
Entpd2	Sense	GCT GGG TGG GCC GGT GGA TAC G	331 bp
	Antisense	ATT GAA GGC CCG GGG ACG CTG AC	
Entpd3	Sense	CGG GAT CCT TGC TGT GCG TGG CAT TTC TT	267 bp
	Antisense	TCT AGA GGT GCT CTG GCA GGA ATC AGT	
Nt5e	Sense	CCC GGG GGC CAC TAG CAC CTC A	403 bp
	Antisense	GCC TGG ACC ACG GGA ACC TT	
Actb	Sense	TAT GCC AAC ACA GTG CTG TCT GG	210 bp
	Antisense	TAC TCC TGC TTC CTG ATC CAC AT	

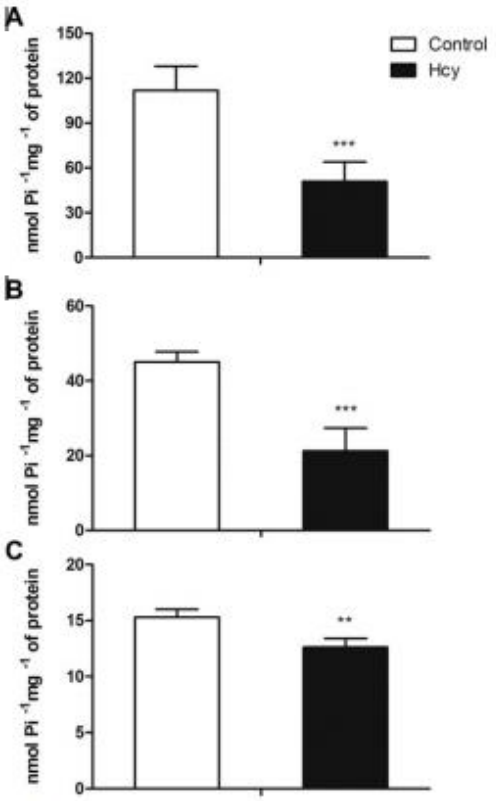

Fig. 1. Effect of chronic homocysteine administration on ATP (A), ADP (B) and AMP (C) hydrolysis in synaptosomal fraction of rat cerebral cortex. Results are expressed as mean ± S.D. for four to six animals in each group. Different from control, $^{**}p < 0.01$; $^{***}p < 0.001$ (Student's *t* test). Hcy – homocysteine.

Statistical analysis

Data were analyzed by Student's *t* test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in synaptosomal fraction from cerebral cortex of adult rats

Fig. 1 demonstrates the effect of chronic mild hyperhomocysteinemia on nucleotide hydrolysis in synaptosomal fraction of rat cerebral cortex. Our results showed that Hcy significantly decreased the hydrolysis of ATP [$t(9) = 6.733$; $p < 0.001$], ADP [$t(9) = 8.596$; $p < 0.001$] and AMP [$t(7) = 5.352$; $p < 0.01$] as compared to control.

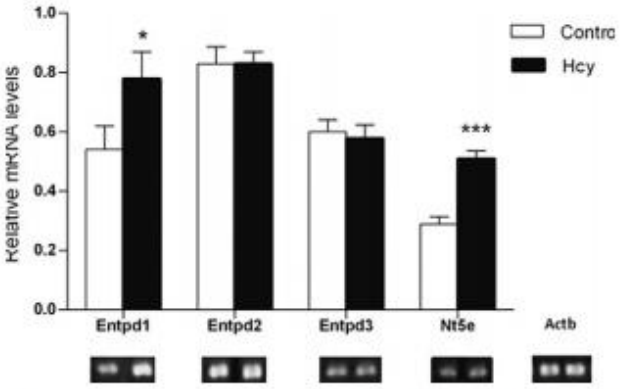

Fig. 2. Gene expression patterns after chronic Hcy treatment for NTPDase1 (Entpd1), NTPDase2 (Entpd2), NTPDase3 (Entpd3), ecto-5′-nucleotidase (Nt5e) and β-actin (Actb) in cerebral cortex of adult rats. Results are expressed as mean ± S.D. for four animals in each group. Different from control, $^{*}p < 0.05$; $^{***}p < 0.001$ (Student's *t* test). Hcy – homocysteine.

Effect of chronic mild hyperhomocysteinemia on E-NTPDases and ecto-5′-nucleotidase gene expression in cerebral cortex of adult rats

As reported in Fig. 2, the chronic mild hyperhomocysteinemia increased Entpd1 gene expression [$t(5) = -3.522$; $p < 0.05$], but not affect Entpd2 and 3 transcript levels [$t(6) = 0.006$; $p > 0.05$; $t(6) = 0.749$; $p > 0.05$, respectively]. However, Nt5e mRNA transcript [$t(6) = -11.751$; $p < 0.001$] was increased in cerebral cortex of rats. We did not perform the E-NTPDase8 gene expression because it is not expressed in cerebral cortex (Bigonnesse et al., 2004).

Effect of chronic mild hyperhomocysteinemia on ATP, ADP, AMP and Ado concentration in CSF of adult rats

Considering that the chronic mild hyperhomocysteinemia alters the activity and expression of ectonucleotidases, we extend our study and investigated the effect of Hcy

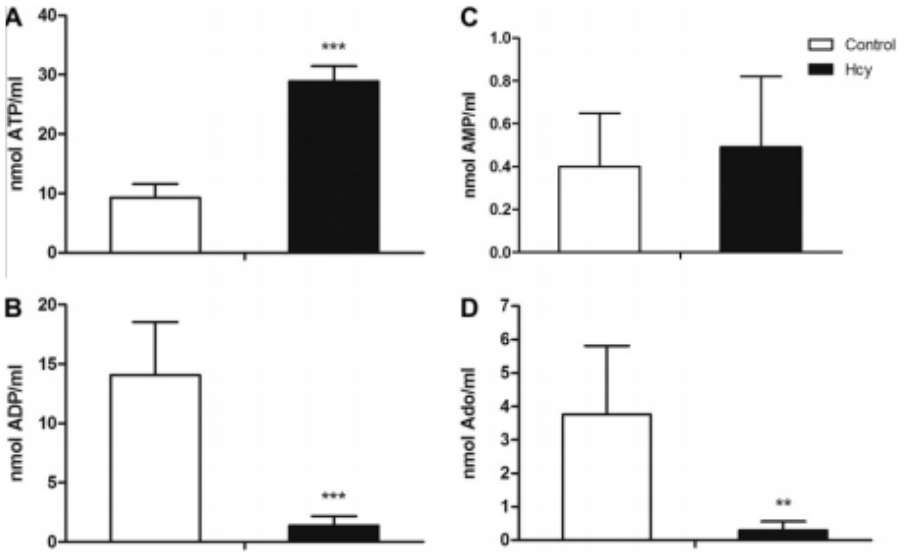

Fig. 3. Effect of chronic homocysteine administration on ATP (A), ADP (B), AMP (C) and Ado (D) concentration in cerebrospinal fluid of adult rats. Results are expressed as mean ± S.D. for four to five animals in each group. Different from control, $^{**}p < 0.01$; $^{***}p < 0.001$ (Student's *t* test). Hcy – homocysteine.

on adenine nucleotides and nucleoside concentration in CSF of adult rats. As can be observed in Fig. 3, ATP concentration was significantly increased in Hcy-treated group [$t(8) = -12.905$; $p < 0.001$] while ADP and Ado concentration were decreased [$t(7) = 6.321$; $p < 0.001$; $t(7) = 3.800$; $p < 0.01$, respectively]. AMP concentration was not affected by Hcy administration [$t(7) = -0.444$; $p > 0.05$].

DISCUSSION

It has been described that Hcy plasma concentration increases with age and that mild hyperhomocysteinemia may be associated with the physiopathology of neurodegenerative diseases (Herrmann et al., 1999; Obeid et al., 2004). Some proposed mechanisms for neurotoxic effects of Hcy involve oxidative stress (Zou and Banerjee, 2005), inflammation (van den Kommer et al., 2010) and glutamatergic excitotoxicity (Shi et al., 2003; Zieminska et al., 2003).

ATP and Ado exert important roles in the CNS, controlling excitatory glutamatergic synapses (Burnstock et al., 2011) and the releasing of neuroinflammatory mediators (Bours et al., 2006; Di Virgilio et al., 2009). In the extracellular space, the concentration of ATP and other nucleotides/nucleosides are tightly controlled by ectonucleotidases (Yegutkin, 2008). In this sense, we recently demonstrated that mild hyperhomocysteinemia decreases ATP, ADP and AMP hydrolysis in lymphocytes and ecto-5′-nucleotidase transcription in mesenteric lymph nodes of adult rats, suggesting that Hcy probably increases ATP and decreases Ado levels, leading to a pro-inflammatory status (Scherer et al., 2012).

In the present study we investigated the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in cerebral cortex of adult rats. Results showed that rats subjected to this model presented a decrease in nucleotide hydrolysis promoted by E-NTPDases and ecto-5′-nucleotidase activities in the synaptosomal fraction of cerebral cortex, which could lead to an increase in ATP and a consequent reduction of Ado levels in the extracellular milieu. Ectonucleotidases are integral membrane proteins (Yegutkin, 2008), therefore, it is plausible to suggest that the oxidative damage in membrane lipids may alter the normal function of these enzymes. Indeed, previous study from our group showed that mild hyperhomocysteinemia increases thiobarbituric acid reactive substances (TBARS) levels, an index of lipid peroxidation (Halliwell, 2006), in cerebral cortex in rats (Scherer et al., 2011). Other study demonstrated that Hcy *in vitro* decreases ATP and ADP hydrolysis in rat platelets probably by oxidative stress since antioxidants prevented such effects (Zanin et al., 2010).

We also investigated whether the chronic mild hyperhomocysteinemia could alter ectonucleotidases gene expression through RT-PCR assays in cerebral cortex of rats. Interestingly, the gene expression pattern of ectonucleotidases presented an increase in mRNA levels of E-NTPDase1 and ecto-5′-nucleotidase. Our results demonstrated that the decrease in ATP, ADP and AMP hydrolysis caused by Hcy treatment was accomplished by an increase in E-NTPDase1 and ecto-5′-nucleotidase mRNA transcript levels. These data suggest that the effect of Hcy on expression of ectonucleotidases could be related to a compensatory mechanism due to decrease in nucleotide hydrolysis caused by mild hyperhomocysteinemia in cerebral cortex of rats.

In order to test whether Hcy could alter extracellular nucleotides and Ado levels, we measured the concentrations of ATP, ADP, AMP and Ado in CSF of adult rats by HPLC. Results showed that ATP was

significantly increased, while ADP and Ado levels were decreased in Hcy-treated rats. Since ATP exerts inhibitory effects on ecto-5′-nucleotidase (Cunha, 2001a), we suggest that the decrease in Ado levels observed in our study may be related to the increase in the ATP levels which may lead to ecto-5′-nucleotidase activity inhibition.

ATP, a common constituent of synaptic vesicles, which is released as a co-transmitter together with classical neurotransmitters, such as glutamate noradrenaline, and acetylcholine (Zimmermann, 1994; Burnstock, 2009) may be neurotoxic when released in high levels into the extracellular space by insults such as ischemia/hypoxia (Lutz and Kabler, 1997; Braun et al., 1998; Melani et al., 2005), inflammation, trauma and stress (Nieber et al., 1999; Le Feuvre et al., 2002). The stimulation of P2X7 receptor by ATP induces multiple cytokine pathways that may coordinate inflammatory responses (Le Feuvre et al., 2002), increases glutamate release (Gu and MacDermott, 1997) and promotes cell death (Adinolfi et al., 2005). Thus, the increase in ATP levels promoted by mild hyperhomocysteinemia in our study may be related to the toxic effects of Hcy on the brain.

At the intracellular level, the metabolism of Hcy and Ado is closely related. In normal conditions, the hydrolysis of SAH by SAH hydrolase produces Hcy and Ado. However, it has been proposed that the increase in Hcy levels promotes the opposite direction of reaction catalyzed by SAH hydrolase, resulting in a decrease in the intracellular Ado concentration and increase in transmembrane Ado concentration gradient. By the action of nucleoside transporter it leads to a decrease in the extracellular Ado levels (Riksen et al., 2003, 2005). Based on the above, we suggest that mild hyperhomocysteinemia may impair both sources of Ado production since the Hcy levels are increased and nucleotide degradation cascade is inhibited. These data corroborate with studies that show that the hyperhomocysteinemia decreases Ado concentration in plasma and renal tissue from rats (Chen et al., 2002) and that Hcy and Ado levels are negatively correlated in rat serum (Bohmer et al., 2010).

Ado regulates many physiological processes, particularly in excitable tissues such as brain (Cunha, 2001b). This nucleoside acts both as a homeostatic transcellular messenger and as a neuromodulator, controlling neurotransmitter release such as glutamate, and neuronal excitability (Barrie and Nicholls, 1993; Fredholm et al., 2005). Ado also presents other functions such as control of metabolism rate of neurons and astrocytes (Haberg et al., 2000; Hammer et al., 2001) vascular resistance (Shin et al., 2000), axonal growth (Rivkees et al., 2001), and analgesic (Johansson et al., 2001) and anticonvulsant properties (reviewed in Fredholm et al., 2005).

Studies have shown that the density of the different Ado receptors is ontogenetically regulated. A decrease in the density of A_1 receptors and an increase in the density of A_{2A} receptors in aged animals have been observed (Cunha et al., 1995, 2001; Lopes et al., 1999; Rebola et al., 2003). Furthermore, in some diseases, such as ischemia, the increase in Ado release could contribute to increase cerebral blood flow and protect cerebral tissue from ischemic insult (Coney and Marshall, 1998). Provided that elevated Hcy levels have been linked to cerebral vascular damage (Faraci and Lentz, 2004; Lee et al., 2005), and that glutamatergic excitotoxicity is associated with hyperhomocysteinemia (Zieminska and Lazarewicz, 2006) it is possible that the decrease in Ado levels could contribute to the cerebral alterations promoted by Hcy. Fig. 4 summarizes the effects of mild hyperhomocysteinemia on extracellular adenine nucleotides metabolism in rat brain, highlighting those processes that were quantified throughout the investigations.

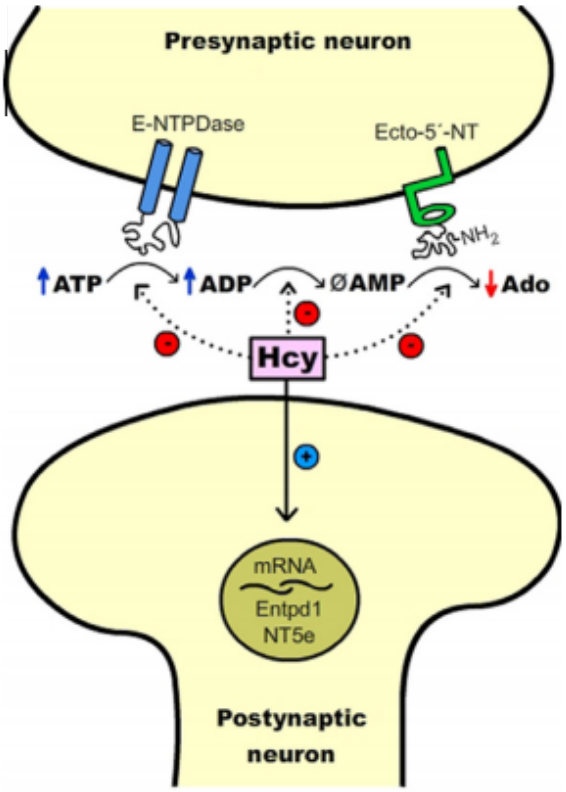

Fig. 4. Summary of the effects of mild hyperhomocysteinemia on extracellular adenine nucleotides metabolism in rat brain, highlighting those processes that were quantified throughout the investigations. Homocysteine (Hcy) inhibits E-NTPDase and ecto-5′-nucleotidase (Ecto-5′-NT) activities and consequently extracellular ATP levels were increased, while ADP and adenosine (Ado) concentrations were decreased. Moreover, Hcy increases E-NTPDase1 (Entpd1) and ecto-5′-nucleotidase (Nt5e) gene expression, suggesting a compensatory mechanism to the decrease in nucleotide hydrolysis.

Since the maintenance of adenine nucleotides and nucleosides levels in the CNS is extremely important to normal brain function since these molecules play a significant role in the pathophysiology of various neurological disorders (Di Virgilio et al., 2009) and that the mild hyperhomocysteinemia is a risk factor for neurodegenerative diseases (Obeid and Herrmann, 2006), our findings lead us to the hypothesis that the

alterations in the ectonucleotidases and the unbalance in ATP and Ado levels might contribute, at last in part, to cerebral damage caused by Hcy.

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Capítulo VI

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Mild Hyperhomocysteinemia Increases Brain Acetylcholinesterase and Proinflammatory Cytokine Levels in Different Tissues

Emilene B. S. Scherer · Samanta O. Loureiro · Fernanda C. Vuaden · Aline A. da Cunha · Felipe Schmitz · Janaína Kolling · Luiz Eduardo B. Savio · Maurício R. Bogo · Carla D. Bonan · Carlos A. Netto · Angela T. S. Wyse

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Abstract Mild hyperhomocysteinemia is considered to be a risk factor for cerebral and cardiovascular disorders and can be modeled in experimental rats. Inflammation has been implicated in the toxic effects of homocysteine. Cholinergic signaling controls cytokine production and inflammation through the "cholinergic anti-inflammatory pathway," and brain acetylcholinesterase activity plays a role in this regulation. The aim of this present study is to investigate the effect of mild chronic hyperhomocysteinemia on proinflammatory cytokine levels in the brain, heart, and serum of rats. Activity, immunocontent, and gene expression of acetylcholinesterase in the brain and butyrylcholinesterase activity in serum were also evaluated. Mild hyperhomocysteinemia was induced in Wistar rats by homocysteine administration (0.03 μmol/g of body weight) twice a day, from the 30th to the 60th days of life. Controls received saline in the same volumes. Results demonstrated an increase in tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and the chemokine monocyte chemotactic protein-1 (MCP-1) in the hippocampus, as well as an increase in IL-1β and IL-6 levels in cerebral cortex. Acetylcholinesterase activity was increased in rats subjected to mild hyperhomocysteinemia in both cerebral structures tested; the immunocontent of this enzyme was also increased in the cerebral cortex and decreased in the hippocampus. Levels of acetylcholinesterase mRNA transcripts were not altered. Peripherally, homocysteine increased TNF-α, IL-6, and MCP-1 levels in the heart and IL-6 levels in serum. Taken altogether, these findings suggest that homocysteine promotes an inflammatory status that can contribute, at least in part, to neuronal and cardiovascular dysfunctions observed in mild hyperhomocysteinemia.

Keywords Mild hyperhomocysteinemia · Cytokines · Gene expression · Acetylcholinesterase · Butyrylcholinesterase

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Introduction

Homocysteine (Hcy), an endogenous sulfur-containing amino acid produced by the methionine metabolism, has recently been recognized as one of the most potent excitatory agents in the central nervous system (CNS) [1, 2]. Elevated circulating levels of Hcy (hyperhomocysteinemia) can be classified according the severity: mild (15–30 μmol/L), moderate (31–100 μmol/L), and severe (>100 μmol/L) [3, 4]. Although severe hyperhomocysteinemia is rare, about 1 in 20 individuals presents mild hyperhomocysteinemia [5], it is a well-established risk factor for several cerebral and cardiovascular disorders, such as epilepsy [6, 7], stroke [8], neurodegenerative and neuropsychiatric diseases [9–12], atherosclerosis, and brain ischemia [13, 14].

The underlying mechanism by which Hcy exerts its toxic effects remains unexplained. In order to research into that mechanism, an experimental model of chronic mild hyperhomocysteinemia in adult rats was developed [15] aiming to

produce plasma levels of Hcy similar to those considered as a risk factor for human cardiovascular and cerebral diseases [16]. Rats submitted to such mild hyperhomocysteinemia presented oxidative damage in cerebral cortex and plasma [15], as well as a decrease in nucleotide hydrolysis in the brain accompanied by an increase in the ectonucleotidase gene expression in the cerebral cortex. Accordingly, ATP levels were significantly increased, while adenosine (Ado) decreased in cerebrospinal fluid of Hcy-treated rats [17]. Since ATP shows proinflammatory properties and Ado is considered an anti-inflammatory compound [18], these results suggest that inflammation might be associated to the adverse effects of mild hyperhomocysteinemia.

Inflammation has been associated with the physiopathology of many neurodegenerative and cardiovascular diseases. An increase in levels of inflammatory proteins in the brain and plasma of Alzheimer's disease and vascular dementia patients has been described [19], and recent studies have shown that inflammatory cytokines play a central role in the pathogenesis of atherosclerosis [20]. Platelet-activating factors (PAFs), reactive oxygen species (ROS), nitric oxide (NO), and various proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), are some of the important inflammation mediators [21, 22], and recent studies of acute and chronic administration of Hcy producing plasma levels similar to those found in severe hyperhomocysteinemia showed greater concentration of cytokines (TNF-α, IL-1β, and IL-6) and chemokine monocyte chemoattractant protein-1 (MCP-1) in serum, hippocampus, and cerebral cortex [23, 24].

The brain cholinergic system is known to modulate several important functions such as learning, memory, cortical organization of movement, and the control of cerebral blood flow [25–28]. Recent studies also suggest the involvement of acetylcholine (ACh) in the inflammation through the "cholinergic anti-inflammatory pathway," defined as neural signals transmitted via the vagus nerve to inhibit the release of peripheral cytokines [29–31]. Along with that, both enzymes that hydrolyze ACh, Acetylcholinesterase (AChE, EC 3.1.1.7) which are highly present in the brain and butyrylcholinesterase (BuChE, EC 3.1.1.8) found in blood serum, pancreas, liver, and SNC [21, 32], have been considered regulators of inflammation since they control ACh action [21].

In the present study, we decided to investigate the effect of chronic mild hyperhomocysteinemia on the proinflammatory cytokine levels in the brain, heart, and serum of rats. The activity, immunocontent, and gene expression of AChE in the cerebral cortex and hippocampus, as well as serum BuChE activity were also evaluated in order to better understand the association between mild hyperhomocysteinemia and inflammation.

Material and Methods

Animals and Reagents

Female Wistar rats (30 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences at the Universidade Federal do Rio Grande do Sul, in Porto Alegre, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in an air-conditioned constant temperature (22±1 °C) colony room, with free access to water and 20 % (*w*/*w*) protein commercial chow. Animal care followed the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1996), and the experimental protocol was approved by the University's Ethics Committee (#19634).

Chronic Mild Hyperhomocysteinemia

DL-Hcy (Sigma-Aldrich®) (0.03 μmol/g of body weight) was administered subcutaneously, twice a day, from the 30th to the 60th day of the life of rats; controls received the same volume of saline solution (0.5 mL/100 g of body weight). Plasma Hcy concentration in rats subjected to such treatment achieved levels similar to those described for the plasma of patients with mild hyperhomocysteinemia (30 μM) [15]. The rats were decapitated 12 h after the last Hcy injection. The cerebral cortex, hippocampus, heart, and serum were used for the biochemical analysis.

Cytokines (TNF-α, IL-1β, and IL-6) and Chemokine CCL2 (MCP-1) Assay

For the acquisition of serum, the whole blood was centrifuged at 1,000×*g* for 5 min, and serum was immediately removed. The cerebral cortex, hippocampus, and heart were homogenized in 1:5 (*w*/*v*) saline solution (0.9 % NaCl). The homogenate was centrifuged at 800×*g* for 10 min at 4 °C, and the supernatant was used in the assays.

TNF-α, IL-1β, IL-6, and MCP-1 levels in the cerebral cortex, hippocampus, heart, and serum were quantified by rat high-sensitivity enzyme-linked immunoabsorbent assays (ELISA) with commercially available kits (Invitrogen®).

AChE Activity Assay

For the AChE assay, the cerebral cortex and hippocampus were homogenized in ten volumes of 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at 1,000×*g*. The supernatants were used for the enzymatic AChE analyses. AChE activity was determined according to the method of Ellman and colleagues [33], with some modifications [34]. Hydrolysis rates were measured at ACh concentration of 0.8 mM in 300 μL assay solution with 30 mM

phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at 25 °C. About 15 μL of cerebral cortex and hippocampus supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). All samples were run in triplicate.

Western Blot Analysis of AChE

Cerebral cortex and hippocampus of rats were homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4 % sodium dodecyl sulphate (SDS). For electrophoresis analysis, samples were dissolved in 25 % (*v*/*v*) of a solution containing 40 % glycerol, 5 % 2-mercaptoethanol, and 50 mM Tris–HCl, pH 6.8, and boiled for 5 min. Total protein homogenate were separated by 10 % SDS–PAGE (50 μg/lane of total protein) and transferred (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20 % methanol, and 0.25 % SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5 % defatted dry milk). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05 % Tween 20 (T-TBS) and then incubated overnight at 4 °C in blocking solution containing AChE antibody (rabbit polyclonal IgG, AChE (H-134), Santa Cruz Biotechnology) diluted 1:1000. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore).

Gene Expression Analysis by Semiquantitative RT-PCR

Analysis of the ache gene expression was performed by a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay [35]. Twelve hours after the last injection of Hcy, the cerebral cortex and hippocampus of rats were isolated for total RNA extraction with the Trizol® Reagent (Invitrogen) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) species were synthesized with the ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following suppliers. cDNA (1 μL) was used as a template for PCR with specific primer for ache. β-actin was used for normalization as a constitutive gene. PCR reactions for ache and β-actin genes were performed in a total volume of 20 μL, containing 0.1 μM primers, 0.2 mM DNTP, 2 mM $MgCl_2$, and 0.5 U Taq DNA Polymerase® (Invitrogen). The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min at the annealing temperature (ache: 55 °C, β-actin: 58.5 °C), and 1 min at 72 °C for 35 cycles. Postextension at 72 °C was performed for 10 min. The amplification products were ache 785 bp and β-actin 210 bp. For each set of PCR reactions, negative control was included. PCR products were analyzed on a 1 % agarose gel containing GelRed® (Biotium) 10× and photographed under UV light. The band intensities were measured by optical densitometry using the freeware ImageJ 1.37 for Windows, and the relative gene expression was determined through the band intensities of ache genes compared to β-actin (ache/β-actin).

BuChE Activity Assay

BuChE activity was determined by the method of Ellman and colleagues [33] with some modifications. Hydrolysis rates were measured at ACh concentration of 0.8 mM in 1 mL assay solution with 100 mM potassium phosphate buffer, pH 7.5, and 1.0 mM DTNB. Fifty microliters of rat-diluted serum was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2 min (intervals of 30 s) at 25 °C. All samples were run in triplicate.

Protein Determination

Protein was measured by the Coomassie Blue method according to Bradford [36] or Lowry and colleagues [37] using bovine serum albumin as the standard.

Statistical Analysis

Data were analyzed by Student's *t* test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant whenever $p<0.05$.

Results

The effect of chronic Hcy administration on cytokine levels (TNF-α, IL-1β, and IL-6) and chemokine CCL2 (MCP-1) in the cerebral cortex and the hippocampus of rats is depicted in Fig. 1. Hcy significantly increased the levels of IL-1β ($p<0.05$) and IL-6 ($p<0.01$) but did not alter TNF-α ($p>0.05$) or MCP-1 levels ($p>0.05$) in the cerebral cortex. However, in the hippocampus, a significant increase in the levels of all cytokines tested (TNF-α ($p<0.01$), IL-1β ($p<0.01$), IL-6 ($p<0.01$), and MCP-1 ($p<0.01$) was observed.

In order to investigate whether mild hyperhomocysteinemia would affect AChE function, we evaluated this enzyme

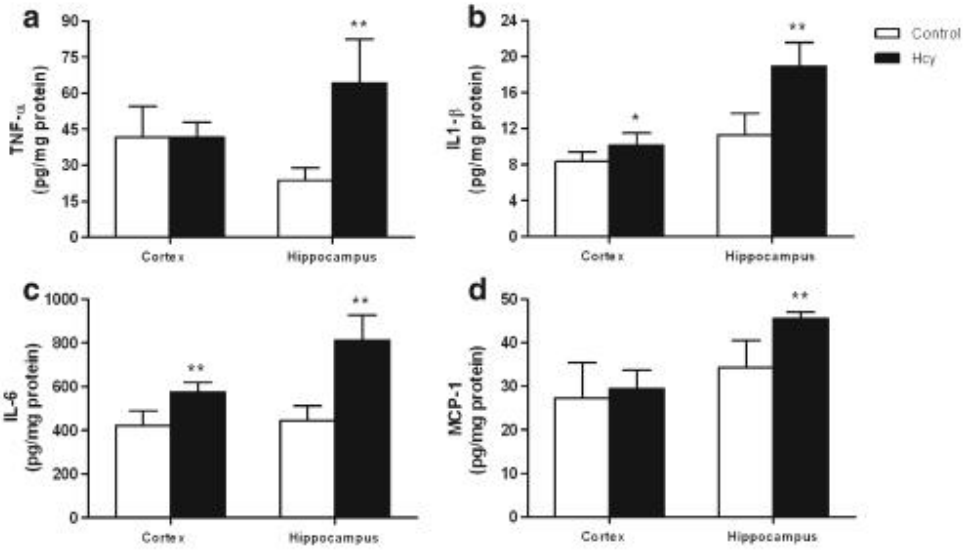

Fig. 1 Effect of chronic homocysteine administration on cytokines (TNF-α, IL-1β, and IL-6) and chemokine CCL2 (MCP-1) levels in the cerebral cortex and hippocampus of rats. Results are expressed as mean±SD for four to five animals in each group. Different from control, *$p<0.05$, **$p<0.01$ (Student's t test). *Hcy* homocysteine, **a** *TNF-α* tumor necrosis factor alpha, **b** *IL-1β* interleukin-1β, **c** *IL-6* interleukin-6, **d** *MCP-1* monocyte chemoattractant protein-1

activity, its immunocontent, and gene expression in the cerebral cortex and hippocampus. The chronic exposure to Hcy caused a significant increase of AChE activity in the cerebral cortex ($p<0.01$) and the hippocampus of rats ($p<0.05$) (Fig. 2). Mild hyperhomocysteinemia also increased the immunocontent of AChE in the cerebral cortex ($p<0.05$) but decreased in the hippocampus ($p<0.01$) (Fig. 3). Quantitative RT-PCR analysis showed that ache gene expression was not altered by Hcy in both brain structures tested ($p>0.05$) (Fig. 4).

The effects of mild hyperhomocysteinemia on proinflammatory cytokine levels in the heart and serum, as well as on BuChE activity in the serum of rats, were also evaluated. It was observed that Hcy significantly increased TNF-α ($p<0.01$), IL-6 ($p<0.05$), and MCP-1 ($p<0.001$) levels in the heart (Fig. 5). Hcy also increased IL-6 levels ($p<0.001$) (Fig. 6) but did not alter TNF-α ($p>0.05$), IL-1β ($p>0.05$), and MCP-1 ($p>0.05$) levels or BuChE activity ($p>0.05$) in the serum of treated rats.

Discussion

Mild hyperhomocysteinemia and inflammation are associated with the development and progression of several cerebral and cardiovascular diseases; however, the mechanism of the relationship between both conditions is unclear. Since CNS interacts dynamically with the immune system to modulate inflammation and ACh has been considered an anti-inflammatory molecule [31], the aim of the present study was to evaluate the proinflammatory cytokines levels in the brain, heart, and serum of rats, as well as the activity, immunocontent, and gene expression of AChE in the cerebral cortex and hippocampus, and BuChE activity in the serum of rats subjected to a model of mild hyperhomocysteinemia.

The proinflammatory cytokines TNF-α, IL-1β, and IL-6 participate prominently in the immune response to pathogens in the periphery and have unique and specific actions on neurons and circuits within the brain [38], being released by macrophages and microglia as part of the early acute phase

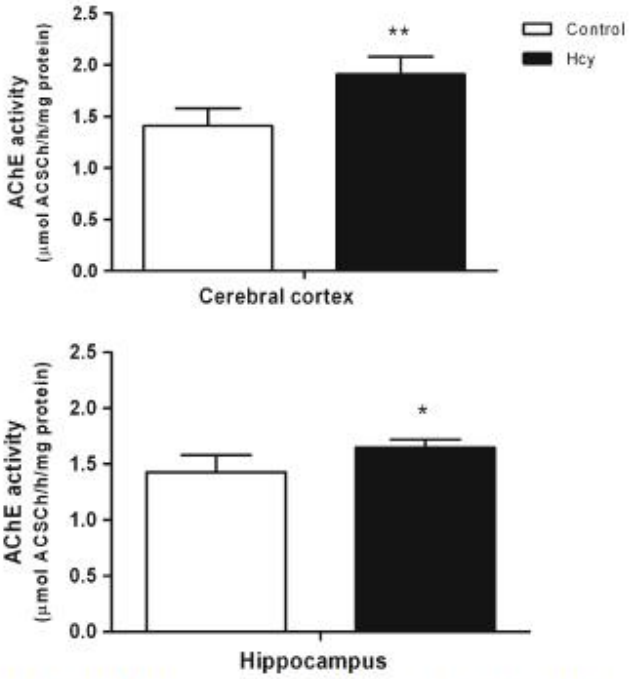

Fig. 2 Effect of chronic homocysteine administration on acetylcholinesterase activity in the cerebral cortex and hippocampus of adult rats. Results are expressed as mean±SD for five to six animals in each group. Different from control, *$p<0.05$, **$p<0.01$ (Student's t test). *Hcy* homocysteine, *AChE* acetylcholinesterase

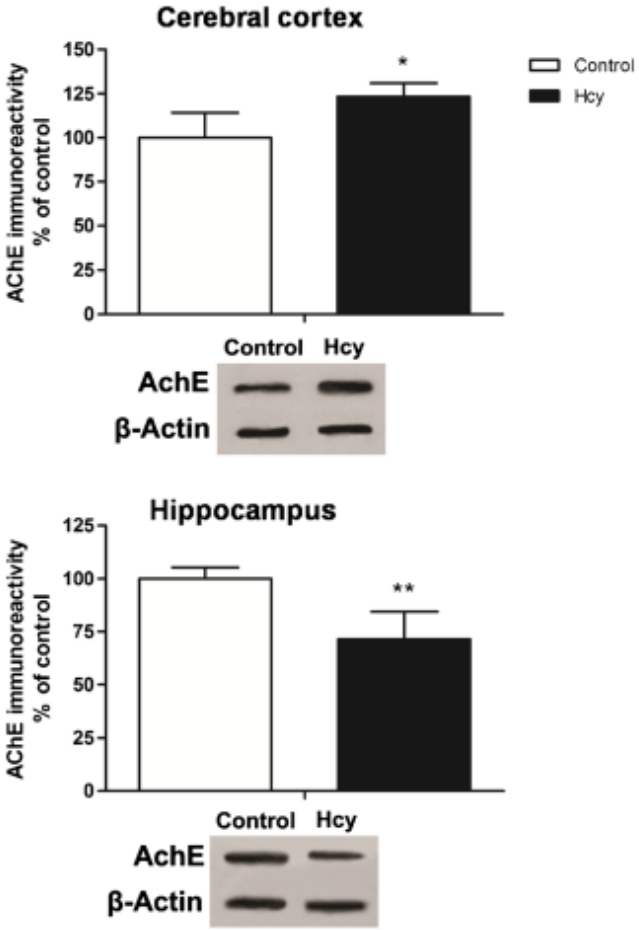

Fig. 3 Effect of chronic homocysteine administration on immunocontent of the acetylcholinesterase in the cerebral cortex and hippocampus of rats. All lanes received equivalent amounts (50 μg) of total protein from cell extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard, since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean±SD for five to six animals in each group. Different from control, $*p<0.05$, $**p<0.01$ (Student's *t* test). *Hcy* homocysteine, *AChE* acetylcholinesterase

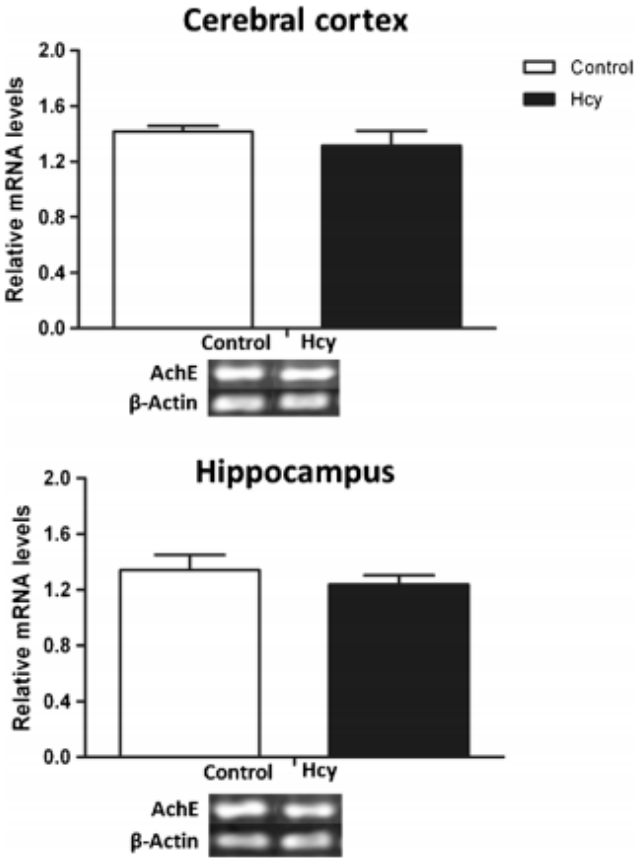

Fig. 4 Gene expression of ache after homocysteine administration in the cerebral cortex and hippocampus of rats. Results are expressed as mean± SD for four to five animals in each group. *Hcy* homocysteine

reaction [39]. TNF-α is the key initiator of immune-mediated inflammation in multiple organ systems including the brain [40], acting synergistically with IL-1β to induce IL-6 expression [41]. In addition to the immune response, these cytokines are suggested to affect neurotransmission, memory modulation, and glucocorticoid function, as well as animal behavior and fear conditioning [38].

Present results show that mild hyperhomocysteinemia increased TNF-α, IL-1β, and IL-6, as well as the chemokine CCL2 (MCP-1) in the hippocampus of rats. In the cerebral cortex, we observed an increase in IL-1β and IL-6 levels. In this context, we previously demonstrated that ATP levels were increased and adenosine was decreased in cerebrospinal fluid of hyperhomocysteinemic rats [17]. ATP released during inflammation presents proinflammatory properties while adenosine, produced by catabolism of ATP, is an anti-inflammatory compound [18]. In addition to that, it has been reported that the stimulation of P2X7 receptor by ATP induces multiple cytokine pathways that may coordinate inflammatory responses [42]. These data suggest that the mild hyperhomocysteinemia promotes a proinflammatory state in the brain.

The "cholinergic anti-inflammatory pathway" regulates the peripheral cytokine response through neural signals transmitted via the vagus nerve [29, 43], and the degeneration of cholinergic neurons may increase the release of proinflammatory cytokine in the periphery system [44]. A similar pathway has been found in the brain of mice, since microglial activation was shown to be regulated by ACh nicotinic receptors [45].

Our results show that chronic mild hyperhomocysteinemia enhances AChE activity in the cerebral cortex and hippocampus of adult rats, suggesting a reduction in the ACh levels leading to a proinflammatory state. Along with that, it has been reported that AChE inhibition reduces microglial production of TNF-α in a hypoxia model [46] and that galantamine, an AChE inhibitor used in the treatment of cholinergic insufficiency and memory loss in Alzheimer's disease, suppresses systemic cytokine levels during endotoxemia [43]. Thus, the increase in the proinflammatory cytokine levels in

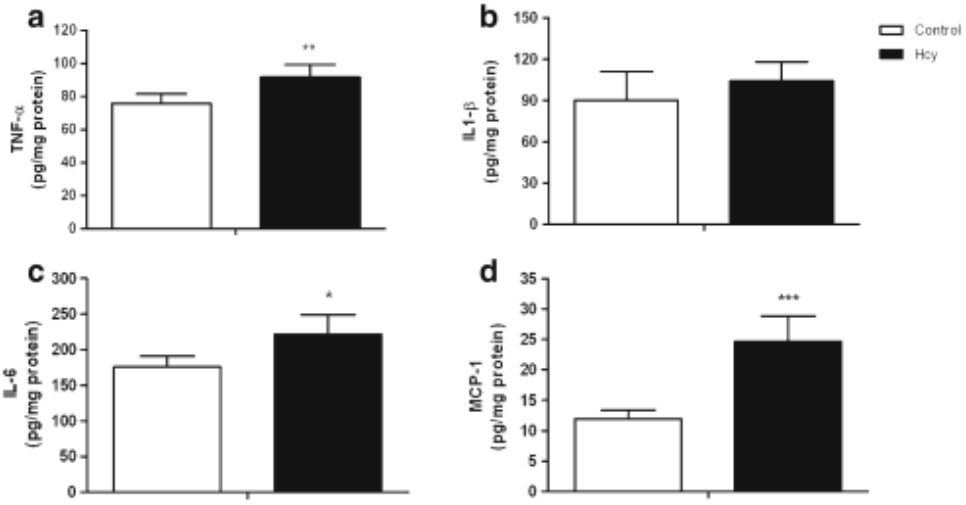

Fig. 5 Effect of chronic homocysteine administration on cytokines (TNF-α, IL-1β, and IL-6) and chemokine CCL2 (MCP-1) levels in the heart of rats. Results are expressed as mean± SD for four to six animals in each group. Different from control, *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's *t* test). *Hcy* homocysteine, **a** *TNF-α* tumor necrosis factor alpha, **b** *IL-1β* interleukin-1β, **c** *IL-6* interleukin-6, **d** *MCP-1* monocyte chemoattractant protein-1

the cerebral cortex and hippocampus caused by mild hyperhomocysteinemia can be associated, at least in part, with the enhancement of AChE activity, since this enzyme hydrolyzes ACh, which is considered an anti-inflammatory molecule that can act by inhibiting the production of proinflammatory mediators [21, 47].

Considering that chronic mild hyperhomocysteinemia increases the AChE activity, the immunocontent and gene expression of this enzyme in the cerebral cortex and hippocampus of adult rats were further studied. Results demonstrate that the immunocontent of AChE was significantly increased in the cerebral cortex and decreased in hippocampus, while the enzyme gene expression pattern was not affected in both cerebral structures tested.

It has been reported that AChE levels are controlled by post-transcriptional regulation [48, 49]. The mechanism by which Hcy changes the AChE immunocontent in the cortex and hippocampus in different forms (increasing the former while decreasing the latter) is not clear. However, it is known

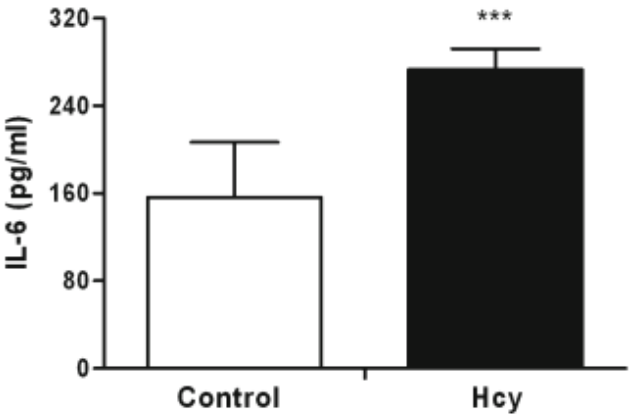

Fig. 6 Effect of chronic homocysteine administration on IL-6 levels in the serum of rats. Results are expressed as mean±SD for five to seven animals in each group. Different from control, ***$p<0.001$ (Student's *t* test). *Hcy* homocysteine, *IL-6* interleukin-6

that AChE isoforms respond differently under toxicological and pathological stress. AChE variants that show indistinguishable enzymatic activity differ in their multimeric assembly and membrane association patterns [49, 50].

It is so plausible to suggest that the stimulation of AChE activity in cerebral cortex can be due to an increment in the content of catalytic subunits, increasing the levels of available enzyme molecules. On the other hand, the decrease of AChE immunocontent in hippocampus could be related to a compensatory response to such augmented enzyme activity.

Studies show that Hcy may promote endothelial dysfunction, leading to the activation of proinflammatory pathways in the vasculature [51, 52]. Hcy stimulates the IL-6 expression in cultured rat aorta vascular smooth muscle cells [53] and induces both mRNA expression and protein secretion of MCP-1 and IL-8 in cultured human aortic endothelial cells [54]. Elevation in the IL-6 levels is closely related to an increased risk of myocardial infarction and coronary diseases [55–58]. Holven et al. [59] showed that the IL-6 levels are increased in hyperhomocysteinemic subjects. Our results are in agreement with these studies, since we observed that mild hyperhomocysteinemia promotes an increase in TNF-α, IL-6, and MCP-1 levels in the heart, as well as increased IL-6 levels in the serum. Taken altogether, these findings support the hypothesis that elevated Hcy levels promote systemic inflammation, which is considered a risk factor to cardiovascular diseases.

Although the physiological role of BuChE is unclear, this enzyme catalyzes the hydrolysis of ACh and thus serves as a coregulator of cholinergic transmission, maintaining the structural and functional integrity of cholinergic pathways [60, 61]. Studies also suggest a relationship between BuChE activity and risk factors for coronary artery disease [62]. In the present study, we observed that BuChE activity was not affected by Hcy treatment in the serum. On the other hand, it is known that BuChE and AChE differ in their kinetic response to

concentrations of ACh. BuChE is less efficient in ACh hydrolysis at low concentrations, but highly efficient at high-substrate concentration when AChE activity is decreased [21]. Thus, the lack of Hcy effect on BuChE activity in the serum can be related to a decrease in the ACh levels by AChE stimulation in the brain, since studies suggest that the peripheral cholinergic system may communicate with the CNS via the vagus nerve [31].

In summary, in the present study, we demonstrated that adult rats submitted to mild hyperhomocysteinemia model presented an increase in the cytokine levels in the brain, heart, and serum. Brain AChE function was also altered by Hcy, suggesting that mild hyperhomocysteinemia may promote a proinflammatory status. These findings provide new basis for the understanding of toxicity mechanisms of Hcy in cerebral, cardiovascular, and peripheral tissues.

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IV. DISCUSSÃO

A hiper-homocisteinemia, caracterizada pelo acúmulo de Hcy na circulação, tem sido considerada um fator de risco para doenças cerebrais e cardíacas embora os mecanismos ainda não estejam completamente elucidados. Estima-se que 5 a 7% da população tenham hiper-homocisteinemia leve uma vez que essa condição pode ser causada, entre outros fatores, por deficiências nutricionais de vitaminas, especialmente do complexo B que estão envolvidas no metabolismo da Hcy, pelo uso de certos medicamentos tais como metotrexato, fenitoína e isoniazida, por doença renal e envelhecimento (Aksoy et al., 2006; Castro et al., 2006; Selhub, 2006; Troen et al., 2008).

Embora haja lacunas entre os modelos animais e as condições humanas que esses modelos tentam representar, os mesmos são muito úteis para a compreensão dos fenômenos envolvidos na patologia de doenças humanas. No nosso grupo de pesquisa já foram desenvolvidos modelos animais para diversos erros inatos do metabolismo tais como: hiperprolinemia (Pontes et al., 1999), fenilcetonúria (Wyse et al., 1995), homocistinúria (Streck et al., 2002), hipermetioninemia (Stefanello et al., 2007) e hipermetioninemia gestacional (Schweinberger et al., 2013).

A hiper-homocisteinemia leve tem sido comumente induzida por modelos de dietas, como a depleção de ácido fólico ou outras vitaminas do complexo B e enriquecimento com Met (Blaise et al., 2007; Fukada et al., 2006). Uma limitação dessas abordagens é que as dietas podem afetar os níveis de vários metabólitos além da Hcy. Para os estudos de função vascular pode ser difícil distinguir as respostas provocadas pela Hcy em relação a outros fatores que são alterados pelas dietas experimentais. Nesses modelos, foi proposto que tanto a deficiência de ácido fólico quanto o excesso de Met pode ter efeitos

adversos sobre a função vascular independentes da Hcy (Doshi et al., 2002; Troen et al., 2003). Além disso, tem sido demonstrado que a Met em elevadas concentrações pode ser tóxica, especialmente para o cérebro (Harvey Mudd et al., 2003). Por outro lado, a deficiência de ácido fólico pode prejudicar outras funções biológicas nas quais ele está envolvido como síntese de ácidos nucléicos e formação de células sanguíneas (Finglas et al., 2003).

O principal objetivo desse trabalho foi desenvolver um modelo animal químico experimental de hiper-homocisteinemia leve em ratos adultos. A dose foi escolhida com o objetivo de induzir, nos animais, concentrações plasmáticas de Hcy semelhantes àquelas consideradas fator de risco para o desenvolvimento de doenças cerebrais e cardíacas.

No capítulo 1, os resultados mostraram que entre 15 a 30 min após a injeção de Hcy o nível plasmático máximo foi de aproximadamente 30 µM, semelhante ao descrito no plasma de pacientes com hiper-homocisteinemia leve (De Bree et al., 2002). Doze horas após a administração, os níveis plasmáticos de Hcy retornaram ao normal. A partir desses resultados, foram calculados os parâmetros farmacocinéticos e determinada a dose para induzir a hiper-homocisteinemia leve crônica (0,03 µmol/g de peso corporal). A queda rápida das concentrações plasmáticas de Hcy ocorre, provavelmente, devido a sua rápida metabolização e excreção (Mudd et al., 2001). Um perfil metabólico semelhante também foi observado em concentrações plasmáticas mais elevadas de Hcy (Streck et al., 2002). Além disso, não podemos descartar a possibilidade de captação da Hcy por outros tecidos tais como cérebro e coração.

O tratamento crônico com Hcy foi realizado do 30^{o} ao 60^{o} dia de vida dos ratos, pois a hiper-homocisteinemia leve é uma condição que afeta

principalmente jovens e adultos (De Bree et al., 2002). O pico plasmático de Hcy após o tratamento crônico foi similar ao encontrado após uma única injeção: aproximadamente 34 µM (15 min) e 32 µM (30 min), retornando ao normal doze horas após a última injeção. Nossos dados também mostraram que animais submetidos a esse modelo não apresentaram qualquer alteração no peso corporal, cerebral ou cardíaco quando comparado com animais tratados com solução salina (controles), indicando que a Hcy não interfere no estado nutricional dos animais. No tecido cerebral não foi possível detectar os níveis de Hcy, entretanto é bem documentado que esse aminoácido atravessa a barreira cérebro-sangue, sendo transportado do plasma para o cérebro e vice-versa por transportadores bidirecionais de membrana (Grieve et al., 1992).

Utilizando esse modelo avaliamos parâmetros de estresse oxidativo em sangue e córtex cerebral de ratos. Primeiramente, demonstramos que a Hcy aumentou os níveis de ERO e TBARS em plasma e córtex cerebral. Esses dados corroboram com estudos mostrando que no plasma a Hcy sofre auto-oxidação do seu grupamento sulfidrila gerando homocistina, dissulfetos mistos e ERO tais como $O_2^{\bullet-}$, H_2O_2 e $OH^{\bullet}$ (Baydas et al., 2006; Loscalzo, 1996; Tyagi, 1998). As lipoproteínas plasmáticas bem como os ácidos graxos poliinsaturados presentes nas membranas cerebrais são suscetíveis ao ataque dessas espécies reativas (Gutteridge, 2001; Mates et al., 1999), conforme evidenciando pelo aumento da peroxidação lipídica em plasma e córtex cerebral de ratos. A oxidação do LDL colesterol por espécies reativas pode contribuir para a fisiopatologia de doenças cardiovasculares (Calmarza et al., 2013). Além disso, alterações em lipídios de membranas podem prejudicar várias funções como transporte, permeabilidade, fluidez e atividade de enzimas (Swapna et al., 2006).

A seguir demonstramos que a hiper-homocisteinemia leve promoveu uma redução nos níveis de nitritos, um produto estável proveniente da auto-oxidação do $NO^{\bullet}$ (Ignarro et al., 1993), em córtex cerebral sem qualquer alteração no plasma. O $NO^{\bullet}$, sintetizado a partir de L-arginina pela ação da enzima óxido nítrico sintase (NOS), é um regulador muito importante de funções vasculares tais como fluxo sanguíneo, agregação plaquetária e inflamação (Forstermann, 2010). Estudos têm demonstrado que a Hcy promove disfunção endotelial através da diminuição nos níveis de $NO^{\bullet}$ ou redução da sua biodisponibilidade (Eberhardt et al., 2000). A hiper-homocisteinemia está relacionada a um acúmulo da dimetilarginina assimétrica (ADMA), um inibidor endógeno da enzima NOS. Além de inibir a produção de $NO^{\bullet}$, a ADMA promove desacoplamento da enzima óxido nítrico sintase endotelial (Lentz, 2005). Devido à sua elevada reatividade, o grupo sulfidrila da Hcy pode reagir com o $NO^{\bullet}$ levando a formação de S-nitroso-homocisteína. Em condições fisiológicas, a formação de S-nitroso-homocisteína fornece proteção às células endoteliais para os efeitos tóxicos da Hcy. Entretanto, o excesso de Hcy gera $O_2^{\bullet-}$ que reage com o $NO^{\bullet}$ para formar $ONOO^{-}$, um potente oxidante (Stamler et al., 1993; Tyagi et al., 2005). A formação de $ONOO^{-}$ reduz a disponibilidade de $NO^{\bullet}$, prejudicando suas funções regulatórias nos vasos sanguíneos. Nesse contexto, podemos sugerir que o aumento na produção do $O_2^{\bullet-}$ pela Hcy leva a formação de $ONOO^{-}$, com consequente redução dos níveis de nitritos observada em córtex cerebral de ratos. Em plasma, embora os níveis de nitritos estejam inalterados, a biodisponibilidade de $NO^{\bullet}$ pode estar diminuída devido à geração de espécies reativas e consequente peroxidação lipídica promovida pela Hcy.

As defesas antioxidantes enzimáticas e não-enzimáticas também foram investigadas. Resultados demonstraram um aumento na razão SOD/CAT enquanto a GPx não foi alterada em eritrócitos e córtex cerebral de ratos. A SOD atua sobre o radical $O_2^{\bullet -}$ dismutando essa molécula a H_2O_2, que pode ser decomposta pela CAT ou GPX. Dessa forma, um equilíbrio entre a atividade coordenada dessas enzimas na detoxificação de espécies reativas é necessário para manter a integridade das biomoléculas. O desequilíbrio observado nesse estudo sugere o acúmulo de H_2O_2 que pode reagir com metais de transição através de reações de Fenton e Haber-Weiss para formar o poderoso radical $OH^{\bullet}$, um potente indutor de peroxidação lipídica (Halliwell and Whiteman, 2004).

O TRAP representa as defesas antioxidantes não enzimáticas presentes nos tecidos ou fluidos biológicos. Demonstramos uma redução significativa nesse parâmetro em plasma e córtex cerebral de ratos hiper-homocisteinêmicos. Esses achados corroboram com o trabalho de Lee e cols. (2007) que demonstraram que a hiper-homocisteinemia diminui as concentrações de vitaminas A e E em plasma de ratos. Um estudo prévio realizado pelo nosso grupo de pesquisa demonstrou que a Hcy *in vitro* (10 µM a 500 µM) reduziu significativamente o TRAP em córtex parietal, pré-frontal e cingulado de ratos (Matte et al., 2004).

No segundo capítulo da presente tese medimos os níveis de Hcy no tecido cardíaco e avaliamos parâmetros de estresse oxidativo em coração e aorta de ratos submetidos ao modelo experimental de hiper-homocisteinemia leve. Nossos dados revelam que após 30 dias de tratamento crônico com Hcy os níveis do aminoácido foram de aproximadamente 5 nmol/g de tecido 30 min após a última injeção e 1,8 nmol/g de tecido 12 h após a última administração.

Nos animais controles os níveis foram de aproximadamente 0,9 nmol/g de tecido. De forma semelhante ao plasma, a concentração cardíaca máxima de Hcy ocorreu 30 minutos após a última administração do aminoácido, entretanto no coração, após 12 h os níveis de Hcy ainda estavam cerca de duas vezes maiores nos animais tratados em relação aos controles. O acúmulo de Hcy no coração pode estar relacionado ao metabolismo mais lento devido à deficiência na transulfuração, desde que no tecido cardíaco, a enzima CBS está ausente gerando uma via de transulfuração incompleta (Finkelstein, 1998).

No coração, as fontes celulares de produção de ERO incluem os miócitos cardíacos, células endoteliais, músculos lisos vasculares e fibroblastos. Nessas células, as ERO podem ser formadas pela mitocôndria, NADPH oxidase, xantina oxidase e desacoplamento da NOS. O miocárdio está equipado com uma variedade de sistemas antioxidantes enzimáticos e não enzimáticos endógenos que são suficientes para neutralizar ERO geradas durante o metabolismo celular. As defesas antioxidantes enzimáticas compreendem a SOD, CAT e GPx. Os mecanismos não-enzimáticos incluem antioxidantes intracelulares, tais como a vitamina E, C e β-caroteno (precursor da vitamina A), a ubiquinona, ácido lipóico, ácido úrico e GSH (Nediani et al., 2011).

Em relação aos parâmetros de estresse oxidativo avaliados no coração de ratos adultos submetidos à hiper-homocisteinemia leve crônica, os resultados demonstram que a produção de ERO, níveis de nitritos, atividades das enzimas antioxidantes, conteúdo de GSH, TBARS e conteúdo de sulfidrilas não foram alterados 12 horas após o término do tratamento com Hcy. Em contraste, o TRAP foi significantemente reduzido, enquanto o conteúdo de carbonilas (marcador de oxidação protéica) foi aumentado no coração de ratos.

O aumento no número de grupos carbonilas está correlacionado com os danos causados às proteínas pelas ERO. Eles podem ser resultado de glicação de proteínas, açúcares, oxidação direta de cadeias laterais de aminoácidos por ERO, entre outros. Além disso, a carbonilação protéica também pode ocorrer através da reação com vários produtos gerados durante a peroxidação lipídica tais como 4-hidroxinonenal, 2-propenal e malondialdeído (Dalle-Donne et al., 2003; Requena et al., 2001). O estresse oxidativo pode causar modificações reversíveis ou irreversíveis nas proteínas sensíveis ao ataque de ERO ou ERN. Modificações reversíveis geralmente ocorrem em resíduos de cisteína das proteínas e podem estar relacionadas a um mecanismo de proteção contra danos irreversíveis e modulação da função protéica (regulação redox). Modificações irreversíveis como carbonilação em resíduos de lisina e arginina são geralmente associadas com perda de função e podem levar à degradação protéica (Dean et al., 1997; Grune et al., 2003). Foi observado que a hiper-homocisteinemia leve crônica promoveu um aumento significativo no conteúdo de carbonilas. Analisando esses resultados, é possível sugerir que durante 30 dias de tratamento alterações oxidativas tenham ocorrido no tecido cardíaco, levando a um consumo das defesas antioxidantes intracelulares e dano protéico. No entanto, o tecido cardíaco foi capaz de se adaptar aos efeitos promovidos pelo Hcy. Corroborando com essa hipótese, estudos demonstram que o coração desenvolve mecanismos de adaptação ao aumento de espécies reativas (Nishizawa et al., 1999).

Na artéria aorta, os níveis de ERO, avaliados através da oxidação do DCFH, não foram alterados, porém os níveis de nitritos foram significativamente diminuídos. A atividade da SOD foi aumentada, sem qualquer alteração na CAT. Observou-se uma redução significativa no TRAP

enquanto os níveis de GSH não foram alterados. Com relação ao dano às biomoléculas, o TBARS e o conteúdo de carbonilas foram significantemente aumentados na aorta de ratos hiper-homocisteinêmicos.

O $NO^{\bullet}$ é neutro, hidrofóbico e capaz de atravessar as membranas, e o $O_2^{\bullet}$ é aniônico em pH neutro. Com base nisso, sugeriu-se que a formação de $ONOO^-$ ocorre predominantemente perto dos locais de produção de $O_2^{\bullet}$ (Alvarez and Radi, 2003). A ativação da SOD observada no presente estudo sugere que a atividade da enzima pode estar aumentada para proteger o vaso arterial de danos causados pelo radical $O_2^{\bullet}$ ou pelo $ONOO^-$, formado pela reação do $O_2^{\bullet}$ com o $NO^{\bullet}$. É importante salientar que o aumento na atividade da SOD não foi acompanhado pelo aumento da CAT, sugerindo um acúmulo de H_2O_2 que pode levar a formação de $OH^{\bullet}$.

Embora pareça controversa a redução no TRAP sem alteração na concentração de GSH, não existe consenso na literatura sobre a redução de GSH em animais com hiper-homocisteinemia moderada ou leve, uma vez que em tais condições não ocorre um bloqueio da via transulfuração que conduz à síntese da GSH, tal como na deficiência severa da enzima CBS ou deficiência de vitamina B6 (Dayal and Lentz, 2007). Desse modo, a redução no TRAP pode ser atribuída à depleção de vitaminas antioxidantes. A peroxidação lipídica e o dano a proteínas observados na aorta podem estar relacionados ao desequilíbrio entre as defesas antioxidantes enzimáticas e a depleção dos antioxidantes não enzimáticos.

Cabe ressaltar que o estresse oxidativo tem sido relacionado a diversas patologias que afetam o SNC e cardiovascular (Karbach et al., 2013; Kovacic and Somanathan, 2012). Nesse contexto, nossos resultados mostrando o estado pró-oxidante promovido pela Hcy em plasma, cérebro, coração e aorta

de ratos submetidos ao modelo experimental de hiper-homocisteinemia leve pode contribuir para os efeitos tóxicos da Hcy sobre esses tecidos.

Alterações na Na^+,K^+-ATPase podem induzir danos importantes na função cerebral uma vez que essa enzima é crucial para a atividade neuronal e captação de neurotransmissores (Blanco, 2005). Considerando que a Na^+,K^+-ATPase é sensível ao ataque por ERO e produtos resultantes da peroxidação lipídica (Dencher et al., 2007), que a inibição da atividade da enzima é encontrada em várias doenças que afetam SNC (Benarroch, 2011), e que a Hcy aumentou a produção de ERO e peroxidação lipídica em córtex cerebral de ratos, nós investigamos no terceiro capítulo o efeito da hiper-homocisteinemia leve sobre a atividade, imunoconteúdo e expressão gênica das subunidades catalíticas (α_1, α_2 e α_3) da Na^+,K^+-ATPase em córtex cerebral e hipocampo de ratos adultos.

Primeiramente, observamos uma redução na atividade da Na^+,K^+-ATPase em ambas as estruturas cerebrais testadas. Estudos prévios *in vitro* realizados no nosso grupo de pesquisa mostram que a Hcy inibiu diretamente a atividade da Na^+,K^+-ATPase em córtices parietal, pré-frontal e cingulado (Matte et al., 2004). Além disso, animais submetidos ao modelo experimental crônico de hiper-homocisteinemia severa apresentaram redução na atividade da enzima em hipocampo (Streck et al., 2003) e córtices parietal, pré-frontal e cingulado (Matte et al., 2004). A redução na atividade da Na^+,K^+-ATPase causada pela hiper-homocisteinemia severa parece ser ocasionada, entre outros fatores, pelo estresse oxidativo, uma vez que a administração de antioxidantes tais como as vitaminas E e C (Wyse et al., 2002) e ácido fólico (Matte et al., 2007) foi capaz de prevenir essa inibição. Com base nesses achados, é plausível sugerir que a inibição na atividade da Na^+,K^+-ATPase

causada pela hiper-homocisteinemia leve observada no presente estudo pode estar associada, pelo menos em parte, à peroxidação lipídica, desde que a enzima está inserida na membrana e qualquer alteração nesse microambiente pode prejudicar sua funcionalidade.

Em relação aos resultados obtidos a partir dos ensaios de determinação do imunoconteúdo das isoformas da Na^+,K^+-ATPase, observamos que houve uma redução significativa no imunoconteúdo das isoformas α_1 e α_2 enquanto a α_3 não foi alterada. O padrão de expressão gênica das isoformas catalíticas não foi afetado pela hiper-homocisteinemia leve em córtex cerebral e hipocampo de ratos. Em conjunto, a redução da atividade enzimática, diminuição do imunoconteúdo das isoformas catalíticas sem alteração da expressão gênica sugere que podem ter ocorrido modificações pós-transcricionais nas isoformas da enzima. Além disso, há evidências mostrando que a quantidade de enzima na membrana plasmática pode ser modificada por alterações na taxa de síntese ou de degradação dos polipeptídeos individuais, bem como pela mobilização de moléculas de enzima do “pool” endossomal para a superfície da célula (Blanco and Mercer, 1998; McDonough and Farley, 1993).

A Na^+,K^+-ATPase desempenha um papel crucial na manutenção das necessidades metabólicas básicas e para as funções especializadas de transmissão do impulso nervoso no SNC. O estabelecimento do potencial de repouso, a captação de neurotransmissores e o efluxo de cálcio estão acoplados a sua atividade. A inibição da enzima pode provocar um aumento da concentração de Na^+ celular, o que pode conduzir a alterações do pH intracelular (via sistema de troca Na:H), ou aumento na concentração intracelular de cálcio e levar a diversos distúrbios cerebrais (Kaplan, 2002).

Em resumo, no terceiro capítulo dessa tese demonstramos que a Hcy diminui a atividade e imunoconteúdo das isoformas da subunidade catalítica da Na^+,K^+-ATPase cerebral. Em adição ao estresse oxidativo, podemos atribuir a diminuição na atividade da Na^+,K^+-ATPase a menor quantidade de moléculas da enzima disponível no córtex e hipocampo de ratos submetidos à hiper-homocisteinemia leve.

Considerando que a Hcy pode promover alterações vasculares através do dano mediado por espécies reativas ou pela indução da resposta inflamatória (Gokkusu et al., 2010), e que o ATP e Ado modulam processos tais como agregação plaquetária, vasodilatação, fluxo coronariano e inflamação (Di Virgilio et al., 2009), no quarto capítulo da presente tese nós avaliamos o efeito da hiper-homocisteinemia sobre a hidrólise de nucleotídeos promovida pelas ectonucleotidases em linfócitos e soro de ratos adultos. A expressão gênica das E-NTPDases e ecto-5'-nucleotidase também foi avaliada em linfócitos.

Os resultados demonstraram que a hidrólise de ATP, ADP e AMP foi significantemente reduzida em linfócitos. Os linfócitos são fundamentais para as respostas imunes humoral e celular (Weinstein et al., 2004). Essas células estão sujeitas à regulação autócrina ou parácrina por nucleotídeos. Durante a ativação, os linfócitos são capazes de liberar ATP, que promove a migração de linfócitos ativados para os locais de inflamação. Altas concentrações de ATP na sinapse imunológica, entre a célula efetora e a célula alvo, podem ativar os receptores $P2X_7$, resultando em lise celular das células alvo. O ATP também estimula a produção de citocinas tais como IL-1β e TNF-α (Bours et al., 2006). Esses efeitos reforçam o papel pró-inflamatório desse nucleotídeo. A Ado, por outro lado, apresenta propriedades anti-inflamatórias. Tem sido demonstrado que a Ado extracelular atenua a resposta proliferativa dos linfócitos T ativados

através da estimulação de receptores A2A (Hoskin et al., 2002; Zhang et al., 2004) e inibe a produção de citocinas pró-inflamatórias tais como IL-12, TNF-α e INFγ (Lappas et al., 2005; Pinhal-Enfield et al., 2003). Yang e cols. (2006) demonstram que em camundongos submetidos à isquemia, a ativação de receptores A2A em linfócitos T CD4 pela Ado preveniu a injúria ao miocárdio por inibir a ativação e acúmulo de linfócitos T CD4 no coração reperfundido.

Tendo em vista a importância da manutenção do equilíbrio e da disponibilidade de nucleotídeos e Ado no meio extracelular, a inibição da hidrólise dessas moléculas pelas ectonucleotidases em linfócitos de ratos hiper-homocisteinêmicos pode levar a um acúmulo de ATP com conseqüente redução na concentração de Ado. Além disso, sabe-se que o metabolismo intracelular da Hcy e da Ado está associado pela reação catalisada pela enzima SAHH (Riksen et al., 2003). O aumento nos níveis de Hcy induz uma queda na Ado extracelular através da captação pelo transportador equilibrativo de nucleosídeo bidirecional. Nesse contexto, estudos têm demonstrado que uma diminuição nos níveis de Ado pode estar associada aos efeitos adversos da hiper-homocisteinemia, uma vez que várias ações vaso-protetoras são atribuídas a esse nucleosídeo, como vasodilatação (Tabrizchi and Bedi, 2001), inibição da agregação plaquetária (Deguchi et al., 1998), e ativação de enzimas antioxidantes (Maggirwar et al., 1994).

A fim de avaliar se a Hcy provoca modificações transcricionais nos ectonucleotidases em linfócitos de ratos, também investigamos os padrões de expressão dessas enzimas. Resultados demonstraram que a transcrição das E-NTPDase1, 2, 3 não foi afetada pela hiper-homocisteinemia leve crônica. Esses dados mostram que a diminuição na hidrólise de ATP e ADP causada pela Hcy não foi acompanhada por alterações nos níveis de RNA mensageiro

transcrito das E-NTPDases. Nesse contexto, é bem conhecido que a expressão gênica é regulada por vários fatores que envolvem a maquinaria celular e vias de transdução de sinal, sendo que a atividade enzimática não pode ser diretamente correlacionada com o padrão de expressão gênica ou com os níveis de proteína, devido à existência de vários eventos pós-transcricionais (Nedeljkovic et al., 2005). Por outro lado, os níveis de RNA mensageiro da ecto-5'-nucleotidase/CD73 foram significativamente diminuídos em linfonodos mesentéricos de ratos hiper-homocisteinêmicos. Tendo em vista que o ATP e ADP podem exercer efeitos inibitórios sobre a ecto-5'-nucleotidase (Cunha, 2001), é possível que a redução na hidrólise de nucleotídeos promovida pela Hcy possa levar ao aumento nos níveis extracelulares de ATP, e consequente inibição da ecto-5'-nucleotidase.

ATP e outros nucleotídeos de adenina podem ser degradados por nucleotidases, tanto na forma ligada à membrana como na forma solúvel, localizada no meio intersticial ou em fluidos corporais (Zimmermann, 2000). Atualmente é aceito que os níveis exógenos de ATP podem estar aumentados em várias doenças inflamatórias, principalmente como consequência da liberação de nucleotídeos a partir de plaquetas, células endoteliais e vasos sanguíneos (Bodin and Burnstock, 1996; Dubyak, 2000; Hantgan, 1984). Esse aumento na concentração de ATP exógeno é geralmente acompanhado por secreção concomitante e/ou clivagem de várias enzimas para o meio extracelular (Yegutkin et al., 2000). Portanto, nucleotidases presentes no soro podem reduzir o excesso de nucleotídeos extracelulares e têm um importante papel importante na manutenção da homeostase purinérgica (Oses et al., 2004).

Nossos resultados também demonstraram que a hidrólise extracelular de ATP, ADP e AMP não foi alterada em soro de ratos. A ausência de efeito da Hcy sobre a hidrólise de nucleotídeos em soro é intrigante, entretanto, Böhmer e cols. (Bohmer et al., 2006) mostraram que a Hcy, *in vitro,* inibe a hidrólise de nucleotídeos em uma faixa de concentração milimolar, e que essa inibição é dependente da concentração. É importante considerar que *in vivo* outros fatores podem afetar a cascata enzimática e que nossos resultados representam concentrações de Hcy na faixa micromolar, semelhantes às consideradas como fator de risco para as doenças cardio e cerebrovasculares (Herrmann and Obeid, 2011; Weiss et al., 2002).

Em resumo, nesse capítulo foi demonstrado que a hiper-homocisteinemia leve experimental diminuiu significativamente a hidrólise extracelular de ATP, ADP e AMP e a expressão gênica da ecto-5'-nucleotidase em linfócitos de ratos adultos. Esses dados sugerem uma ativação do processo inflamatório, através do aumento na concentração de ATP e diminuição da Ado extracelular.

A fim de dar continuidade aos nossos estudos e considerando que as enzimas ectonucleotidases são amplamente distribuídas no cérebro, e os nucleotídeos estão envolvidos na neuromodulação, o quinto capítulo consistiu na avaliação do efeito da hiper-homocisteinemia leve crônica sobre as atividades das ectonucleotidases e expressão gênica em córtex cerebral de ratos. As concentrações de ATP, ADP, AMP e Ado em líquor de ratos também foram avaliadas por HPLC.

Os resultados mostraram uma diminuição na hidrólise de nucleotídeos pela ação catalítica das ectonucleotidases em sinaptossomas obtidos de córtex cerebral de ratos. A redução na hidrólise pode ser explicada por alterações na

membrana ocasionadas pela Hcy, visto que a atividade das ectonucleotidases é suscetível a mudanças nas propriedades das membranas nas quais elas estão ancoradas (Wang et al., 1998; Yegutkin, 2008). Nós demonstramos neste trabalho que a Hcy promoveu aumento de TBARS, um marcador de peroxidação lipídica, em córtex cerebral de ratos. Desse modo, é possível sugerir que a inibição na atividade das ectonucleotidases em córtex cerebral pode estar relacionada, pelo menos em parte, ao dano oxidativo promovido pela hiper-homocisteinemia leve. Corroborando com essa hipótese, Zanin e cols. (2010) demonstraram que a Hcy, adicionada ao meio de incubação, inibiu a hidrólise de ATP e ADP em plaquetas de ratos nas concentrações de 20 a 500 µM. A adição de trolox, um antioxidante, foi capaz de prevenir tal alteração.

Com relação à expressão gênica da ectonucleotidases, observamos um aumento nos níveis de RNA mensageiro para os transcritos da E-NTPDase1 e ecto-5'-nucleotidase em córtex cerebral de ratos. O fato de termos observado a redução na hidrólise de nucleotídeos sugere um aumento nos níveis de ATP e redução nos níveis de Ado no meio extracelular. Desse modo, o aumento na expressão gênica dessas enzimas pode estar associado a um mecanismo compensatório para regular os níveis dessas moléculas. Com a finalidade de investigar nossa hipótese, avaliamos as concentrações de ATP, ADP, AMP e Ado em líquor de ratos hiper-homocisteinêmicos. Resultados mostraram que os níveis de ATP foram significantemente aumentados, enquanto ADP e Ado diminuídos em líquor de ratos.

O ATP é considerado uma importante molécula sinalizadora no SNC. Como neurotransmissor, ele é liberado para o meio extracelular através de vesículas pré-sinápticas juntamente com outros neurotransmissores como ACh, noradrenalina, glutamato, serotonina e GABA (Burnstock, 2004; Zimmermann,

2008). Em condições patológicas como isquemia, trauma e inflamação, esse nucleotídeo pode ser liberado por neurônios, microglia, células endoteliais e astrócitos ativados. A estimulação do receptor $P2X_7$ pelo ATP leva à ativação da microglia, produção de ERO e aumento da secreção de citocinas pró-inflamatórias, tais como IL-1β, TNF-α e IL-6 (Skaper et al., 2010; Sperlagh et al., 2006). Recentemente, a estimulação do receptor $P2X_7$ tem sido relacionada com a neuroinflamação em muitas doenças do SNC, incluindo a doença de Alzheimer, epilepsia e esclerose múltipla. O tratamento com inibidores desse receptor reduz a neuroinflamação induzida experimentalmente em modelos animais dessas doenças (Kim et al., 2010; Peng et al., 2009; Ryu and McLarnon, 2008; Sperlagh et al., 2006). Além do seu papel na inflamação, parece que o ATP extracelular aumenta a liberação de glutamato (Gu and MacDermott, 1997) e promove morte celular (Adinolfi et al., 2005) através da estimulação de receptores $P2X_7$.

A Ado extracelular também funciona como uma molécula sinalizadora de lesão celular exercendo, na maioria das vezes, ações contrárias as do ATP (Bours et al., 2006). Através da ativação de receptores A2A, a Ado promove imunossupressão por induzir a elevação intracelular de cAMP (Sitkovsky and Ohta, 2005). A Ado regula vários processos no SNC, tais como controle do metabolismo de neurônios e astrócitos, resistência vascular e inflamação. Tem sido demonstrado o efeito protetor desse nucleosídeo após o insulto isquêmico através da melhora no fluxo sanguíneo (Coney and Marshall, 1998) e bloqueio da liberação de neurotransmissores, principalmente o glutamato (Sakamoto et al., 2004). Suas principais ações anti-inflamatórias incluem a redução na liberação de citocinas pró-inflamatórias tais como IL-12 e TNF-α (Hasko et al.,

1998; Hasko et al., 2000; Khoa et al., 2001) e estímulo à produção da IL-10, uma citocina anti-inflamatória (Hasko et al., 1996).

Existem duas principais fontes de Ado extracelular no SNC: liberação de Ado através de transportadores equilibrativos de nucleosídeo bidirecionais e conversão extracelular de nucleotídeos de adenina pelas ectonucleotidases (Latini and Pedata, 2001). A diminuição nos níveis de Ado extracelular evidenciado em nosso estudo pode ser um somatório da redução intracelular desse nucleosídeo devido ao aumento nos níveis de Hcy, associado à inibição da hidrólise extracelular pelas ectonucleotidases. Nossos dados corroboram com estudos mostrando que a Hcy reduz as concentrações de Ado e prejudica suas ações celulares. Chen e cols. (2002) demonstraram uma redução de 50% na concentração de Ado endógena induzida pela discreta elevação da Hcy plasmática. O efeito vasodilator da Ado, reduzido pela Hcy, foi completamente restaurado pelo inibidor de captação de nucleosídeo (dipiridamol) em pacientes com homocistinúria (Riksen et al., 2005).

Tendo em vista a importância do equilíbrio nos níveis de nucleotídeos e Ado para o funcionamento do SNC, os resultados desse capítulo sugerem que a inibição na hidrólise de nucleotídeos em córtex cerebral e o desequilíbrio nos níveis extracelulares de ATP e Ado em líquor de ratos submetidos ao modelo experimental de hiper-homocisteinemia leve, podem estar associados aos danos cerebrais causados pela Hcy.

No capítulo 6, avaliamos o efeito da hiper-homocisteinemia leve experimental sobre os níveis de citocinas pró-inflamatórias (TNF-α, IL-1β e IL-6) e da quimiocina MCP-1 em córtex cerebral, hipocampo, coração e soro de ratos adultos. Resultados demonstram que em hipocampo os níveis de todas as citocinas testadas estão aumentados. Em córtex cerebral, houve um

aumento significativo nos níveis de IL-1β e IL-6 no grupo tratado com Hcy em relação ao grupo controle.

As citocinas pró-inflamatórias desempenham papéis pleiotróficos. No SNC atuam na plasticidade sináptica (Ben Achour and Pascual, 2010), neurotransmissão (Dunn et al., 1999), transdução de sinal nuclear (Song et al., 2009) e neurogênese (Monje et al., 2003), assim como nas respostas inflamatórias. A atividade normal de citocinas pró-inflamatórias pode reduzir o agravamento do processo inflamatório, restaurando assim a homeostase no SNC (Glezer et al., 2007). Entretanto, em altos níveis essas moléculas podem desencadear neurotoxicidade. No cérebro, as citocinas podem ser produzidas pela microglia, célula com função imune residente do SNC que quando ativada secreta uma grande variedade de citocinas pró-inflamatórias e fatores neurotóxicos como ERO e ERN (Lerouet et al., 2002; Liu and Hong, 2003). Além disso, elas também podem ser produzidas por neurônios ou por células imunocompetentes da periferia (Banks, 2005; Woodroofe, 1995).

Nos capítulos anteriores, mostramos que a Hcy promove estresse oxidativo em córtex cerebral além de aumentar a concentração extracelular de ATP e diminuir a de Ado no líquor de ratos. Considerando que as citocinas pró-inflamatórias podem ser produzidas em resposta ao estresse oxidativo (Halliwell and Gutteridge, 2007) ou ao desequilíbrio nos níveis de ATP e Ado, é plausível sugerir que esses achados podem estar estreitamente relacionados. Corroborando com nossos dados, vários estudos têm mostrado que a Hcy promove um estado pró-inflamatório. Foi demonstrada a ativação de células endoteliais em cultura expostas à Hcy, o que resulta em um aumento na expressão de quimiocinas (Poddar et al., 2001) e moléculas de adesão (Koga et al., 2002; Postea et al., 2006; Silverman et al., 2002). Além disso, estudos

prévios realizados no nosso grupo de pesquisa demonstraram que a hiper-homocisteinemia severa aumentou a concentração de citocinas pró-inflamatórias em cérebro e sangue de ratos neonatos (da Cunha et al., 2010; da Cunha et al., 2012).

Estudos têm demonstrado o importante papel do sistema colinérgico no processo inflamatório. A inflamação periférica é controlada, entre outros, pela "via colinérgica anti-inflamatória", que envolve a inibição da resposta imune inata através da liberação de ACh pelo nervo vago. Através dessa via, a liberação de citocinas por monócitos e macrófagos é suprimida (Rosas-Ballina and Tracey, 2009; Tracey, 2002). Esse mecanismo também foi demonstrado no SNC. Em cultura de células microgliais, o pré-tratamento com ACh inibiu a produção de TNF-α induzida pelo LPS (Shytle et al., 2004).

Dando continuidade ao nosso estudo, avaliamos o efeito da hiper-homocisteinemia leve sobre a atividade, imunoconteúdo e expressão gênica da AChE. Resultados demonstraram que a Hcy aumentou significativamente a atividade da AChE no córtex cerebral e no hipocampo de ratos adultos. Desde que a ACh é hidrolisada pela AChE, esses achados sugerem uma redução nos níveis de ACh, o que pode contribuir, pelo menos em parte, para o estado pró-inflamatório promovido pela Hcy. Nesse contexto, em um modelo de hipóxia, a inibição da AChE reduziu a produção de TNF-α (Wang et al., 2010). O fármaco galantamina, inibidor da AChE usado no tratamento da insuficiência colinérgica e perda de memória durante a doença de Alzheimer, suprimiu os níveis de citocinas sistêmicas durante a endotoxemia (Pavlov et al., 2009).

Nossos resultados também demonstraram que o imunoconteúdo da AChE foi significativamente aumentado no córtex cerebral e diminuído no hipocampo, enquanto o padrão de expressão gênica da enzima não foi afetado

pela hiper-homocisteinemia leve em ambas as estruturas cerebrais testadas. Tem sido demonstrado que os níveis da AChE são controlados por regulação transcricional e pós-transcricional (Kaufer et al., 1998; Shohami et al., 2000). O mecanismo pelo qual Hcy alterou o imunoconteúdo AChE por vias opostas no córtex e no hipocampo não está claro, porém, sabemos que as isoformas neuronais da AChE respondem de maneira diferente sob estresse toxicológico e patológico. Variantes da enzima, que mostram atividade enzimática indistinguível, ainda diferem na sua expressão e padrões de associação à membrana (Meshorer and Soreq, 2006; Shohami et al., 2000). No presente estudo, é plausível sugerir que a estimulação da atividade da AChE no córtex cerebral pode estar associada ao aumento na quantidade de moléculas de enzima disponível. Por outro lado, a diminuição no imunoconteúdo da enzima em hipocampo sugere uma resposta compensatória ao aumento da atividade da AChE nessa estrutura cerebral.

A Hcy pode promover disfunção endotelial, o que pode levar à ativação de vias pró-inflamatórias na vasculatura (Liu et al., 2008; Weiss, 2005). A Hcy promove a estimulação da expressão de IL-6 em cultura de células de músculo liso de aorta de ratos (Zhang et al., 2006) e induz a expressão e a secreção de MCP-1 e IL-8 em células endoteliais de aorta humana cultivadas (Poddar et al., 2001). Nossos resultados demonstram que a Hcy aumentou significativamente os níveis de TNF-α, IL-6 e MCP-1 no coração de ratos. No soro, os níveis de IL-6 estavam aumentados após o tratamento crônico, sugerindo que a hiper-homocisteinemia leve promove inflamação sistêmica.

Estudos sugerem que a elevação nos níveis de IL-6 está estreitamente relacionada com um aumento do risco de infarto do miocárdio e as doenças coronárias (Harris et al., 1999; Lindmark et al., 2001; Ridker et al., 2000).

Pacientes com insuficiência cardíaca congestiva apresentam uma correlação entre os elevados níveis plasmáticos de IL-6 e TNF-α e o prejuízo na vasodilatação endotélio-dependente da artéria e veia braquial (Katz et al., 1994; Vanderheyden et al., 1998). Nesse contexto, o aumento das citocinas no coração e da IL-6 em soro de ratos hiper-homocisteinêmicos pode contribuir para desenvolvimento de doenças cardíacas. Corroborando com nossos achados, Holven e cols. (2006) mostraram que os níveis de IL-6 estão aumentados em pacientes com hiper-homocisteinemia.

Outra enzima importante na hidrólise de ACh é a BuChE. Essa enzima está presente de forma mais abundante no soro e embora o seu papel exato na transmissão colinérgica não esteja claro, ela é muito importante na manutenção da integridade estrutural e funcional das vias colinérgicas (Mesulam et al., 2002). Os resultados do presente trabalho demonstraram que a atividade dessa enzima não foi alterada no soro de ratos submetidos à hiper-homocisteinemia leve. Sabe-se que BuChE e AChE diferem em sua resposta cinética de acordo com as concentrações de ACh. A BuChE é menos eficiente na hidrólise de ACh em concentrações baixas, mas altamente eficiente em concentrações elevadas, onde a AChE é inibida (Das, 2007). Assim, a falta de efeito na atividade BuChE pode estar relacionada com um decréscimo nos níveis de ACh por estimulação da AChE cerebral, uma vez que estudos indicam que o sistema colinérgico periférico se comunica com o central através do nervo vago (Rosas-Ballina and Tracey, 2009). Um estudo prévio, *in vitro*, sugere que a atividade BuChE em soro só sofre alteração em altas concentrações de Hcy. Demonstramos que a Hcy não alterou a atividade enzima nas concentrações de 10 µM e 100 µM, porém inibiu significativamente a atividade da BuChE na concentração de 500 µM em soro de ratos de 8, 15 e

60 dias de vida (Scherer et al., 2007). Nos estudos *in vivo*, também foi observada uma diminuição na atividade da enzima na hiper-homocisteinemia severa, cujos níveis plasmáticos de Hcy estão na faixa de 400 µM a 500 µM (Stefanello et al., 2005).

Em conjunto, os resultados deste trabalho mostram que a homocisteína induz um estado oxidativo e inflamatório central e periférico além de prejudicar a funcionalidade de enzimas cerebrais importantes. Considerando que a hiper-homocisteinemia leve é prevalente na população e tem sido considerada um fator de risco para o desenvolvimento de doenças cerebrais e cardíacas, o presente modelo experimental pode ser útil para a investigação da patofisiologia de doenças causadas pela homocisteína e estratégias de prevenção.

Um resumo dos principais resultados observados nessa Tese encontram-se no Anexo 1.

V. CONCLUSÕES

1. Foi desenvolvido um modelo químico experimental de hiper-homocisteinemia leve em ratos adultos, cujas concentrações plasmáticas de Hcy foram similares àquelas consideradas fator de risco para o desenvolvimento de doenças cerebrais e cardiovasculares (aproximadamente 30 µM). Utilizando esse modelo observamos que:
2. A Hcy aumentou a produção de ERO em plasma e córtex cerebral, mas não alterou em coração e aorta;
3. Houve uma redução nos níveis de nitritos em córtex cerebral e aorta. Em plasma e coração de ratos esse parâmetro não foi alterado;
4. A hiper-homocisteinemia leve promoveu um desequilíbrio nas defesas antioxidantes enzimáticas e peroxidação lipídica em plasma, córtex cerebral e aorta de ratos. Em coração esses parâmetros não foram afetados pela Hcy;
5. Houve uma redução nas defesas antioxidantes não enzimáticas em plasma, córtex cerebral, coração e aorta de ratos;
6. A hiper-homocisteinemia leve promoveu um aumento no conteúdo de carbonilas em coração e aorta de ratos;
7. A atividade e o imunoconteúdo das isoformas α_1 e α_2 da Na^+,K^+-ATPase foram diminuídas em córtex cerebral e hipocampo de ratos. A expressão gênica da enzima não foi alterada em ambas as estruturas cerebrais testadas;
8. A hiper-homocisteinemia leve promoveu uma diminuição na hidrólise de ATP, ADP e AMP em linfócitos e em sinaptossomas obtidos de córtex cerebral de ratos. Em soro, tal parâmetro não foi alterado;
9. A expressão da ecto-5'-nucleotidase foi diminuída em gânglios linfáticos mesentéricos e aumentada em córtex cerebral de ratos;

10. Houve um aumento na expressão da E-NTPDase1 em córtex cerebral de ratos;
11. Ratos tratados cronicamente com Hcy apresentaram concentrações aumentadas de ATP e diminuídas de ADP e Ado em líquor;
12. Houve um aumento nos níveis de citocinas pró-inflamatórias: TNF-α, IL-1β, IL-6 e da quimiocina MCP-1 em hipocampo; IL-1β e IL-6 em córtex cerebral; TNF-α, IL-6 e MCP-1 no coração e IL-6 em soro de ratos;
13. A hiper-homocisteinemia leve promoveu uma estimulação da atividade da AChE em córtex cerebral e hipocampo de ratos;
14. O imunoconteúdo da AChE foi aumentado em córtex cerebral e diminuído no hipocampo de ratos;
15. A atividade da BuChE não foi alterada pela Hcy em soro de ratos adultos.

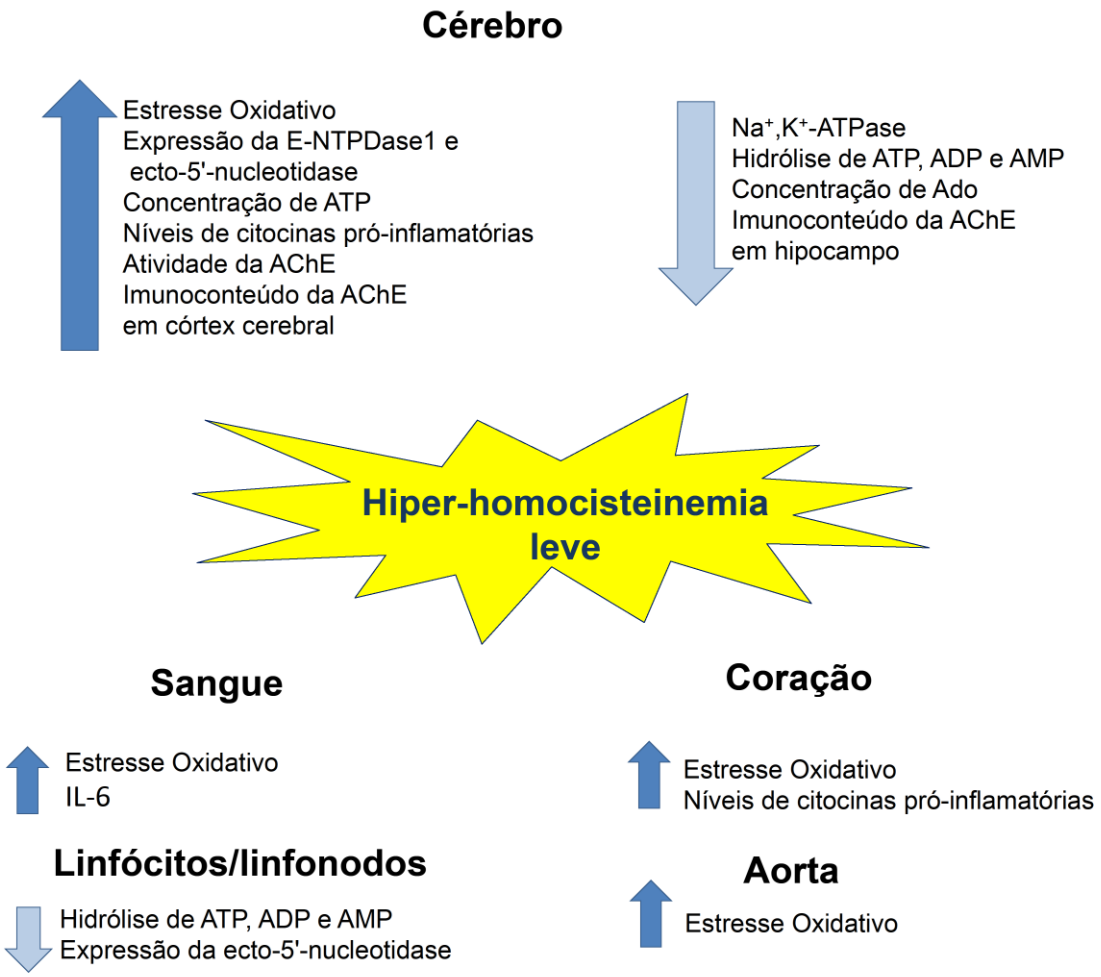
CONCLUSÃO GERAL

Tendo em vista a importância do uso de modelos animais para investigar os mecanismos envolvidos nas doenças humanas, que a hiper-homocisteinemia leve é recorrente na população e tem sido considerada um fator de risco para doenças cerebrais e cardíacas, destacamos a relevância do desenvolvimento do nosso modelo para o estudo das alterações teciduais causadas pela Hcy.

Os resultados do presente estudo mostram, em conjunto, que a hiper-homocisteinemia leve causa uma série de alterações bioquímicas e moleculares no SNC e periferia, tais como indução de estresse oxidativo, alterações nas enzimas cerebrais Na^+,K^+-ATPase, ectonucleotidases e AChE, desequilíbrio nas concentrações de nucleotídeos e Ado em líquor e aumento nos níveis de citocinas pró-inflamatórias que podem contribuir, pelo menos em parte, para os efeitos nocivos da Hcy ao SNC e cardiovascular.

VI. ANEXOS

Anexo 1



VI. PERSPECTIVAS

Avaliar os seguintes parâmetros em ratos adultos submetidos à hiper-homocisteinemia leve experimental:

- Atividade e expressão das ectonucleotidases em coração e plaquetas;
- Função mitocondrial e parâmetros de metabolismo energético em cérebro e coração;
- Captação do glutamato e o imunoconteúdo dos transportadores de glutamato (GLAST e GLT-1) em córtex cerebral e hipocampo;
- O imunoconteúdo das frações citoplasmática e nuclear da subunidade p65 do NF-κB no córtex cerebral, hipocampo e coração;
- O imunoconteúdo do CD11b, marcador de microglia ativada, no córtex cerebral e hipocampo;
- Memória espacial e aversiva;
- Morfologia em tecido cerebral, cardíaco e vascular.

VII. REFERÊNCIAS BIBLIOGRÁFICAS

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