Universidade Federal do Rio Grande do Sul
Instituto de Ciências Básicas da Saúde
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

AVALIAÇÃO DOS EFEITOS NEURO E HEPATOTÓXICOS DA HOMOCISTEÍNA EM RATOS UTILIZANDO UMA ABORDAGEM BIOQUÍMICA E COMPORTAMENTAL: PAPEL ANTIOXIDANTE DO ÁCIDO FÓLICO

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Orientadora: Profª Drª Angela Terezinha de Souza Wyse

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"Toda a nossa ciência, comparada com a realidade, é primitiva e infantil

e, no entanto, é a coisa mais preciosa que temos."

Albert Einstein

SUMÁRIO

PARTE I

RESUMO

A homocistinúria é um erro inato do metabolismo bioquimicamente caracterizado pela deficiência da enzima cistationina β-sintase, o que promove um bloqueio na via de transulfuração da homocisteína, e consequente acúmulo tecidual desse aminoácido. Clinicamente os pacientes apresentam retardo mental, crises convulsivas, alterações vasculares, oculares, esqueléticas e hepáticas. Embora a hiper-homocisteinemia seja uma característica marcante da homocistinúria, o aumento nos níveis de homocisteína tem sido detectados em uma série de doenças neurodegenerativas, cérebro e cardiovasculares, bem como hepáticas. Nesse contexto, o objetivo geral da presente tese foi avaliar o efeito da hiper-homocisteinemia sobre parâmetros comportamentais e bioquímicos (estresse oxidativo, metabolismo energético e captação de glutamato) em cérebro, sangue e fígado de ratos. Também investigamos o papel neuroprotetor do ácido fólico. A hiper-homocisteinemia crônica reduziu as defesas antioxidantes enzimáticas e não-enzimáticas, bem como aumentou o dano ao DNA e o nível de espécies reativas ao ácido tiobarbitúrico em córtex parietal. As defesas antioxidantes sanguíneas também foram afetadas, e o dano ao DNA observado em córtex foi reproduzido em células nucleadas sanguíneas. A administração crônica de homocisteína resultou em danos oxidativos no fígado, reduzindo as defesas antioxidantes e o conteúdo de grupamentos tiólicos, bem como aumentando a peroxidação lipídica. O tecido hepático apresentou ainda alterações histológicas, demonstradas pela presença de infiltrado inflamatório, fibrose e redução no conteúdo de glicogênio/glicoproteínas. As hiper-homocisteinemias aguda e crônica alteraram a atividade da citocromo *c* oxidase possivelmente devido a alterações oxidativas; bem como afetaram o sistema glutamatérgico, reduzindo a captação de glutamato e o imunoconteúdo dos transportadores gliais em córtex parietal de ratos. O tratamento com ácido fólico preveniu as modificações oxidativas em córtex parietal e sangue de ratos, bem como as alterações na atividade da citocromo *c* oxidase. A inibição da Na^+,K^+-ATPase em córtex parietal e o déficit mnemônico espacial em ratos hiper-homocisteinêmicos também foram prevenidos pelo tratamento concomitante com ácido fólico, através de um mecanismo independente da redução dos níveis plasmáticos e cerebrais de homocisteína, e possivelmente relacionado à sua atividade antioxidante. A administração aguda de homocisteína resultou em déficit na consolidação da memória aversiva em ratos, associado à redução no imunoconteúdo de BDNF hipocampal. Esses efeitos foram prevenidos pelo pré-tratamento com ácido fólico. Em resumo, nossos resultados indicam que a homocisteína induz estresse oxidativo em córtex parietal, sangue e fígado de ratos; o que pode estar correlacionado com as demais alterações observadas nessa tese, tais como disfunção mitocondrial, redução na captação de glutamato e déficit mnemônico. Nossos resultados sugerem um efeito neuroprotetor do ácido fólico, prevenindo as alterações bioquímicas e comportamentais induzidas pela administração de homocisteína, possivelmente via ação antioxidante. Acreditamos que nossos achados possam colaborar para o esclarecimento dos mecanismos citotóxicos da homocisteína, bem como apoiar a avaliação da terapia com ácido fólico em pacientes hiper-homocisteinêmicos.

ABSTRACT

Homocystinuria, an inborn error of metabolism, is biochemically characterized by cystathionine β-synthase deficiency and homocysteine tissue accumulation. Patients frequently present mental retardation, seizures, vascular, skeletal, ocular, and hepatic complications. Moreover, hyperhomocysteinemia has emerged as an important risk factor for neurodegenerative, vascular and hepatic diseases. In this context, the main objective of this doctoral thesis was to evaluate the effect of hyperhomocysteinemia on behavioral and biochemical parameters (oxidative stress, energy metabolism, and glutamate uptake) in brain structures, blood and liver of rats. We also investigated the neuroprotective role of folic acid. Chronic hyperhomocysteinemia reduced enzymatic and non-enzymatic antioxidant defenses, as well as increased DNA damage and the level of thiobarbituric acid reactive substances in parietal cortex of rats. Antioxidant defenses were also altered in blood of hyperhomocysteinemic rats, who presented increased DNA damage in blood nucleated cells. Chronic homocysteine administration induced oxidative stress in liver of rats, reducing antioxidant defenses and total thiol content, as well as increasing lipid peroxidation. Histological analyses revealed inflammatory infiltration, fibrosis and reduced glycogen/glycoprotein content in hepatic tissue. Acute and chronic hyperhomocysteinemia resulted in changes in cytochrome *c* oxidase activity, as well affected the glutamatergic system, inhibiting the glutamate uptake and reducing glial transporters immunocontent in parietal cortex. Folic acid administration prevented the oxidative alterations elicited by homocysteine in parietal cortex and blood of rats, as well the modifications on cytochrome *c* oxidase activity. Na^+,K^+-ATPase inhibition in parietal cortex and the spatial mnemonic deficit in hyperhomocysteinemic rats were prevented by folic acid concurrent administration, by a mechanism independent of homocysteine plasmatic and cerebral concentration reduction, and possible dependent of its antioxidant potential. Acute homocysteine administration induced a significant deficit in consolidation of aversive memory, associated to a reduction in hippocampal BDNF immunocontent. Folic acid pretreatment prevented both effects. In summary, our results showed that homocysteine administration induced oxidative stress in parietal cortex, blood, and liver of rats; which could be related to biochemical and behavior alterations found in this thesis, such as mitochondrial dysfunction, reduction in glutamate uptake, and mnemonic deficit. Our data suggest a neuroprotective effect of folic acid, preventing the biochemical and behavior alterations elicited by homocysteine, possible by its antioxidant properties. We believe that our data could collaborate for the explanation of homocysteine cytotoxicity mechanisms, as well support the evaluation of folic acid therapy in hyperhomocysteinemic patients.

LISTA DE ABREVIATURAS

ADP - difosfato de adenosina

ALT- alanina aminotransferase

AMPA - ácido α-amino-3-hidróxi-5-metil-4-isoxazol-propiônico

AST - aspartato aminotransferase

ATP - trifosfato de adenosina

BDNF - fator neurotrófico derivado do encéfalo

BHMT - betaína homocisteína metiltransferase

BHT - di-terc-butil metil fenol

CAT - catalase

CBS - cistationina β-sintase

CGL - cistationina γ-liase

CK - creatina quinase

COX – citocromo *c* oxidase

CREB - elemento de ligação responsivo ao AMPc

e^- - elétron

EAAC - carreador de aminoácidos excitatórios

EAAT - transportador de aminoácidos excitatórios

EIM - erros inatos do metabolismo

Erk - quinase regulada por sinal extracelular

ERO - espécies reativas de oxigênio

$FADH_2$ - flavina adenina dinucleotídeo (forma reduzida)

G - proteína G

GLAST - transportador glial de glutamato

Gln - glutamina

GLT-1 - transportador glutamato-aspartato dependente de Na^+

GLUT - transportador de glicose

GPx - glutationa peroxidase

GR - glutationa redutase

GSH - glutationa (forma reduzida)

GSSG - glutationa (forma oxidada)

HCU - homocistinúria

Hcy - homocisteína

HNE - 4-hidroxinonenal

H_2O_2 - peróxido de hidrogênio

HPLC - cromatografia líquida de alta eficiência

HPLC/MS-MS - HPLC acoplado à espectrometria de massas em tandem

IL - interleucina

IP_3 - inositol trifosfato

Lac - lactato

LDH - lactato desidrogenase

LDL - lipoproteínas de baixa densidade

LTM - memória de longa duração

LTP - potenciação de longa duração

MAPK - proteína quinase ativada por mitógeno

MAT - metionina adenosiltransferase

MCP-1 - proteína quimiotática de monócitos 1

MDA - malondialdeído

Met - metionina

MMP - metaloproteinases

MS - metionina sintase

NADH - nicotinamida adenina dinucleotídeo (forma reduzida)

NADPH - nicotinamida adenina dinucleotídeo fosfato (forma reduzida)

NADPH oxidase - nicotinamida adenina dinucleotídeo fosfato oxidase

NFκB - fator nuclear kappa B

NMDA - *N*-metil-D-aspartato

$NO^{\bullet}$ - óxido nítrico

$NO_2^{\bullet}$ - dióxido de nitrogênio

$O_2^{\bullet -}$ - superóxido

$OH^{\bullet}$ - hidroxil

PARP - poli (ADP-ribose) polimerase

PAS - ácido periódico de Schiff

PGK - fosfoglicerato quinase

PI3K - fosfatidil-inositol 3 quinase

PKA - proteína quinase A

PKC - proteína quinase C

PKG - proteína quinase G

Pi – fosfato inorgânico

PPi - pirofosfato inorgânico

PLCγ - fosfolipase C γ

PLP - piridoxal fosfato

Q - ubiquinona

$RO_2^{\bullet}$ - peroxil

SAH - S-adenosil homocisteína

SAHH - S-adenosil homocisteína hidrolase

SAM - S-adenosilmetionina

SDH - succinato desidrogenase

SNC - sistema nervoso central

SOD - superóxido dismutase

STM - memória de curta duração

TAR - reatividade antioxidante total

TIMP-1 - inibidor de MMP-1

TNF-α - fator de necrose tumoral α

Trk - tirosina quinase

TBARS - substâncias reativas ao ácido tiobarbitúrico

TRAP - potencial antioxidante total não-enzimático

V_m - potencial de membrana

I. INTRODUÇÃO

1. Erros inatos do metabolismo

Erros inatos do metabolismo (EIM) são doenças multifatoriais, majoritariamente autossômicas recessivas, que abrangem alterações genéticas e bioquímicas. São caracterizados pela síntese de produtos gênicos alterados, geralmente enzimas, porém a síntese de receptores, hormônios, fatores de coagulação e hemoglobinas anormais também caracteriza bioquimicamente diversos EIM. Essas desordens implicam num fenótipo químico alterado, como consequência do bloqueio de vias metabólicas onde essas enzimas anômalas estão inseridas (Scriver et al., 2001).

Os primeiros estudos envolvendo EIM foram desenvolvidos por Archibald Garrod, no início do século XX, com a descrição da alcaptonúria. Atualmente, o livro "The Metabolic & Molecular Bases of Inherited Disease" lista mais de 500 EIM, apresentando incrementos sucessivos a cada edição. Os EIM podem ser clinicamente classificados em duas categorias: (1) doenças que afetam um sistema anatômico ou funcional, ou ainda um único órgão, e (2) doenças nas quais a lesão bioquímica afeta uma via metabólica, que é comum em diversos órgãos ou é individual de um órgão, entretanto os efeitos são sistêmicos. A segunda categoria pode ainda ser dividida em 3 subtipos, individualizando o diagnóstico: (2.1) doenças que alteram a síntese ou catabolismo de moléculas complexas, tais como desordens lisossomais, peroxissomais, e alterações no processamento e tráfego intracelular de proteínas secretórias; (2.2) erros inatos do metabolismo intermediário, caracterizados pela intoxicação aguda e progressiva causada pelo acúmulo de compostos tóxicos em decorrência do bloqueio metabólico, incluindo aminoacidopatias, acidúrias orgânicas, defeitos congênitos do ciclo da uréia e intolerância a carboidratos; e (2.3) doenças caracterizadas por "deficiência energética", onde ocorre um distúrbio na produção ou utilização de energia,

como resultado de um EIM intermediário que atinge o fígado, miocárdio, músculo ou cérebro; incluindo defeitos na glicogenólise, gliconeogênese, acidemia lática congênita, defeitos na oxidação de ácidos graxos e na cadeia respiratória mitocondrial. Apesar da freqüência individual dessas doenças ser baixa, em conjunto atingem 1:1000 nascidos vivos (Scriver et al., 2001).

2. Homocistinúria

A homocistinúria (HCU) é um EIM dos aminoácidos sulfurados, associado à deficiência da enzima cistationina β-sintase (CBS; EC 4.2.1.22). Essa doença metabólica foi reportada pela primeira vez em 1969 por McCully, que identificou homocistina na urina de uma paciente com alterações vasculares avançadas. Os achados ateroscleróticos verificados em pacientes homocistinúricos, relatados por McCully, foram pioneiros em associar elevadas concentrações plasmáticas de Hcy e a deficiência das vitaminas do complexo B (McCully, 1969).

Essa desordem autossômica recessiva é geneticamente heterogênea, podendo ser causada por 92 diferentes mutações no gene que codifica a enzima CBS. A HCU apresenta uma prevalência mundial média variando de 1:200.000 à 1:335.000 nascidos vivos (Mudd, Levy e Kraus, 2001), entretanto diversos estudos indicam que esses valores foram subestimados. No Catar, essa prevalência alcança 1:1800, a mais alta identificada até o presente momento, devido à organização tribal dessa população, onde ocorrem muitos casamentos consanguíneos que propagam uma mutação na enzima CBS. Além disso, o evoluído sistema de diagnóstico empregado, através da cromatografia líquida de alta eficiência (HPLC) acoplado à espectrometria de massas em tandem (HPLC/MS-MS), possibilita o diagnóstico preciso em larga escala desse EIM (Zschocke et al., 2009).

Bioquimicamente, a HCU é caracterizada pelo acúmulo tecidual de homocisteína (Hcy), acompanhado pela elevação dos níveis de seu precursor, o aminoácido essencial metionina (Met), assim como da redução dos produtos da via de transulfuração da Hcy, cistationina e cisteína. Além disso, ocorre a eliminação de homocistina, um metabólito formado por duas moléculas de Hcy ligadas por uma ponte dissulfeto, na urina, a característica que dá nome à doença. As concentrações plasmáticas normais de Hcy variam de 5-15 μM, podendo atingir 500 μM na doença, enquanto a concentração plasmática normal de Met de 35 μM pode alcançar 2000 μM em pacientes homocistinúricos não tratados. As altas concentrações sanguíneas encontradas nos pacientes refletem a deficiência enzimática intracelular. Pacientes portadores de HCU apresentam uma considerável redução na atividade da enzima CBS em fígado, fibroblastos, cérebro, linfócitos, células coletadas de líquido amniótico e vilosidades coriônicas (Mudd, Levy e Kraus, 2001).

Os pacientes afetados por essa doença invariavelmente apresentam alterações no sistema nervoso central (SNC) e vascular, além de anormalidades oculares, esqueléticas e hepáticas. Dentre os sinais e sintomas mais frequentes estão o retardo mental, distúrbios psiquiátricos, crises convulsivas, aterosclerose, tromboembolismo, ectopia do cristalino, miopia, osteoporose, esteatose hepática e uma variedade de deformações ósseas. Esses efeitos têm sido relacionados às altas concentrações teciduais de Hcy observadas nos pacientes (Mudd, Levy e Kraus, 2001).

O diagnóstico da HCU é dado através da combinação de sinais clínicos e das avaliações bioquímica e molecular. A história familiar e os dados clínicos do paciente orientam a investigação para os EIM, entretanto esse conjunto de doenças muitas vezes apresenta sintomas semelhantes, dificultando o diagnóstico baseado nos dados observacionais. Em seguida, é realizada uma avaliação bioquímica da urina, plasma e

biópsia tecidual do paciente. O teste de triagem para HCU consiste na identificação da homocistina na urina do paciente, a qual reage no teste do cianeto-nitroprussiato. Esse método apresenta uma série de interferentes, portanto, não é conclusivo, sendo necessária a identificação de Hcy e seus metabólitos no plasma dos pacientes. A detecção e a quantificação plasmáticas dos metabólitos da Hcy podem ser realizadas por eletroforese, cromatografia de troca iônica, HPLC, e mais recentemente HPLC/MS-MS. Essas metodologias também são utilizadas para o monitoramento da eficácia do tratamento empregado em pacientes homocistinúricos. A confirmação do diagnóstico de HCU é dada através de ensaios diretos da atividade da enzima CBS em biópsia de fígado, cultura de fibroblastos da pele ou linfócitos estimulados por fitoemaglutinina obtidos do paciente. O sucesso do tratamento de EIM em geral, incluindo a HCU, depende do diagnóstico precoce. Neste sentido, pode-se realizar o diagnóstico pré-natal através da medida dos metabólitos da Hcy no líquido amniótico, ou pela determinação da atividade da enzima CBS em cultura de células livres do líquido amniótico (Fowler e Jakobs, 1998; Mudd, Levy e Kraus, 2001; Mudd et al., 2003).

A conduta terapêutica utilizada geralmente envolve uma dieta reduzida em Met, além da suplementação com cisteína e vitaminas do complexo B (piridoxina/B_6, cobalamina/B_{12} e ácido fólico/B_9). Nesse contexto, os pacientes homocistinúricos são classificados como responsivos, intermediários e não responsivos à piridoxina, o que está estritamente relacionado à mutação da CBS presente em cada indivíduo, e norteia o tratamento que será utilizado. A dieta com restrição de Met é amplamente utilizada, e permite a redução dos níveis plasmáticos de Hcy, desde que esse aminoácido essencial origina a Hcy durante seu catabolismo celular. Tendo em vista a função da cisteína como precursora da glutationa (GSH), um dos mais importantes antioxidantes cerebrais, faz-se necessário utilizar uma dieta suplementada com cisteína.

As vitaminas são importantes precursores de coenzimas utilizadas no metabolismo da Hcy. Os pacientes responsivos à piridoxina recebem doses diárias dessa vitamina, que podem ultrapassar 1g, o que tem mostrado um controle relativo dos níveis plasmáticos de Hcy, além de um atraso no aparecimento dos sintomas da doença; entretanto a utilização prolongada dessa vitamina tem sido associada ao aparecimento de danos neurológicos. O ácido fólico tem sido empregado em alguns pacientes na dose de 5-10 mg/dia, em regimes que incluem a piridoxina em pacientes responsivos. A cobalamina é menos utilizada nos protocolos de terapia, e geralmente é administrada duas vezes ao ano. A betaína, que participa da via de remetilação da Hcy no fígado, também tem sido administrada a alguns pacientes, em especial aos denominados não-responsivos à piridoxina, porém as altas doses empregadas (6-9 g/dia) e o odor corporal causado por esse composto limitam sua utilização. Essas condutas terapêuticas têm por objetivo controlar os níveis plasmáticos da Hcy e seus metabólitos, retardando a evolução das complicações neurológicas e periféricas, principalmente vasculares, as quais geralmente são responsáveis pelo óbito dos pacientes (Walter et al., 1998; Mudd, Levy e Kraus, 2001).

3. Homocisteína

A Hcy é um aminoácido sulfurado não-protéico derivado da Met, cujo metabolismo está demonstrado na figura 1. O catabolismo da Met, que é obtida da dieta e da degradação de proteínas endógenas, tem um papel fundamental nos processos de metilação molecular. A Met é convertida à *S*-adenosilmetionina (SAM) pela enzima metionina adenosiltranferase (MAT, EC 2.5.1.6), recebendo um grupo adenosil do ATP. A SAM é o principal doador de grupos metil presente no meio biológico, sendo convertida à *S*-adenosil homocisteína (SAH) por diversas metiltransferases, específicas para cada substrato, tal como o DNA, a fosfatidiletanolamina, o guanidinoacetato e

alguns neurotransmissores. A SAH é hidrolisada a Hcy e adenosina pela S-adenosil homocisteína hidrolase (SAHH, EC 3.3.1.1). Nesse ponto, o catabolismo da Hcy pode seguir duas vias: (1) a remetilação à Met, onde a Hcy recebe um grupo metil proveniente do N^5-metiltetraidrofolato, em uma reação catalisada pela metionina sintase (MS, EC 2.1.1.13), ou da betaína, em uma reação catalisada pela betaína homocisteína metiltransferase (BHMT, EC 2.1.1.15); ou (2) a transulfuração à cisteína, onde a Hcy sofre condensação com a serina, produzindo cistationina, em uma reação catalisada pela enzima CBS, que utiliza como coenzima o piridoxal fosfato (PLP), e, no passo seguinte, a cisteína é formada através da reação de clivagem catalisada pela cistationina γ-liase (CGL, EC 4.4.1.1). A rota de remetilação é amplamente distribuída no organismo, enquanto que a via de transulfuração da Hcy tem distribuição limitada, participando do catabolismo da Hcy principalmente no fígado, rins, intestino delgado, pâncreas e cérebro (Fowler, 1997; Brosnan et al., 2004; Finkelstein, 2007).

O metabolismo da Hcy é dependente de uma ingestão adequada de vitaminas, as quais são precursoras de coenzimas que atuam nessa via. O PLP, derivado da piridoxina, é o grupo prostético da CBS e da CGL, ambas pertencentes à via de transulfuração da Hcy. A metilcobalamina e o N^5-metiltetraidrofolato, coenzimas da MS, são derivados da cobalamina e do ácido fólico, respectivamente, e participam da via de remetilação da Hcy (Fowler, 1997; Mudd, Levy e Kraus, 2001; Brosnan et al., 2004).

A concentração total de Hcy no plasma é composta por um conjunto de espécies redox, sendo que a forma oxidada ligada a proteínas é a predominante, perfazendo 70-80% do total da Hcy plasmática, enquanto que a forma oxidada livre (composta por um misto de dissulfetos) encontra-se numa proporção de aproximadamente 20%. A Hcy totalmente reduzida, que é a forma ativa, contribui muito pouco com a concentração

total desse aminotiol, cerca de 1-4%, entretanto na HCU não tratada as altas concentrações de Hcy promovem um aumento desproporcional nos níveis de Hcy reduzida, podendo alcançar 20 µM em pacientes com concentração total de 150 µM, sugerindo que a capacidade de ligação protéica para a Hcy foi ultrapassada (Mudd, Levy e Kraus, 2001; Perna, Ingrosso e De Santo, 2003; Brosnan et al., 2004).

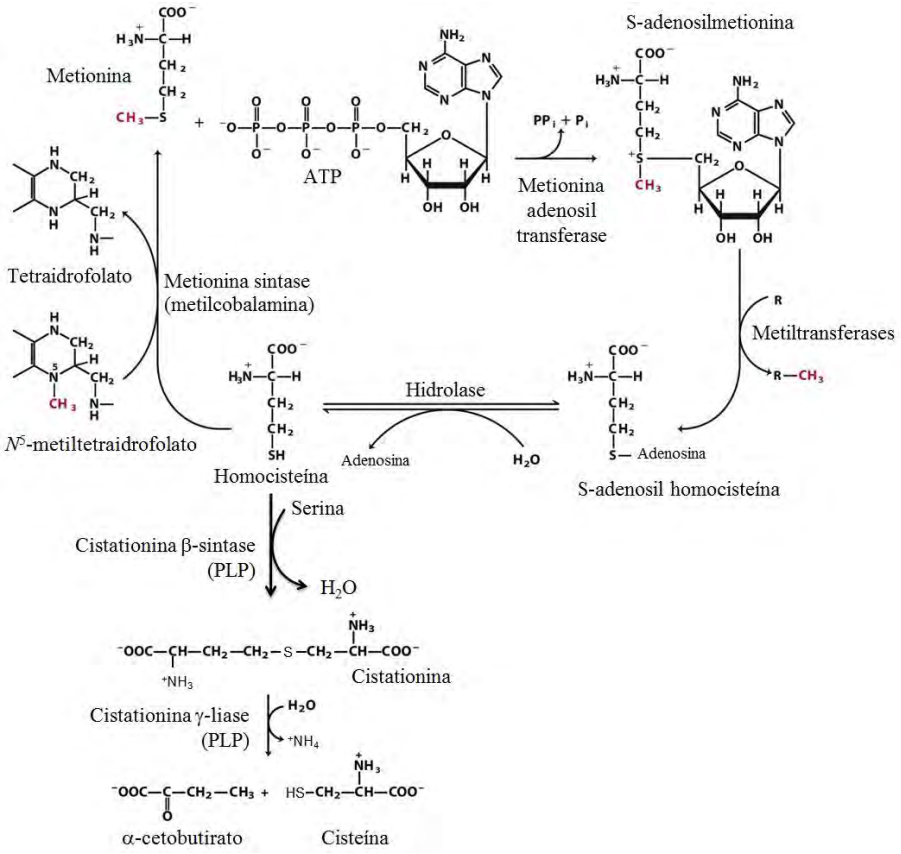

Figura 1. Metabolismo da homocisteína (adaptado de Cox e Nelson, 2001). As coenzimas encontram-se entre parênteses, abaixo das enzimas que catalisam a reação. ATP, trifosfato de adenosina; PLP, piridoxal fosfato; Pi, fosfato inorgânico; PPi, pirofosfato inorgânico

3.1. Aspectos tóxicos da homocisteína

Embora a hiper-homocisteinemia seja um achado bioquímico característico da HCU (Mudd, Levy e Kraus, 2001), essa condição metabólica também tem sido demonstrada em pacientes acometidos de doenças neurodegenerativas (Diaz-Arrastia, 2000; Mattson, Kruman e Duan, 2002), psiquiátricas (Sachdev, 2004; Bottiglieri, 2005), vasculares (Clarke et al., 1991; Welch e Loscalzo, 1998; Faraci e Lentz, 2004) e

hepáticas (Bosy-Westphal et al., 2001; Adinolfi et al., 2005; Ventura et al., 2005). Em adição, a hiper-homocisteinemia tem sido destacada como um fator de risco importante no déficit cognitivo observado em pacientes idosos (Sachdev, 2004; Kado et al., 2005), associado à observação de atrofias hipocampal e cortical identificadas em estudos de imagem (Sachdev, 2005).

Nosso grupo de pesquisa tem demonstrado uma relação consistente entre a administração de Hcy e o prejuízo na memória em modelos animais submetidos às tarefas de esquiva inibitória (Reis et al., 2002) e labirinto aquático de Morris (Streck et al., 2004). Corroborando com esses dados, Bernardo e cols. (2007) mostraram que camundongos transgênicos, super-expressando peptídeo β-amilóide, e alimentados com uma dieta que induziu hiper-homocisteinemia apresentaram déficit na memória espacial avaliada no labirinto aquático de Morris. Baydas e cols. (2005) correlacionaram positivamente a hiper-homocisteinemia e o estresse oxidativo em hipocampo de ratos ao prejuízo na memória espacial, testada no labirinto aquático. Nesse contexto, Rabaneda e cols. (2008) recentemente demonstraram que a Hcy inibe a proliferação de células progenitoras neurais da zona subventricular e do giro denteado do hipocampo de camundongos, áreas cerebrais relacionadas ao aprendizado e memória, através de um mecanismo dependente da inibição da via da quinase regulada por sinal extracelular (Erk, EC 2.7.11.24) 1/2.

Diversos estudos publicados na última década têm apresentado hipóteses que visam elucidar os mecanismos pelos quais a Hcy exerce sua toxicidade, no entanto, ainda não existe um consenso na literatura, considerando a variedade de sistemas afetados por esse aminoácido. Entre os mecanismos de neurotoxicidade mais estudados estão a excitotoxicidade glutamatérgica, o desequilíbrio das reações de metilação celular causado pelo acúmulo de SAH, um potente inibidor das metiltransferases; e as

alterações vasculares (Troen, 2005). Considerando a primeira hipótese, a Hcy possui propriedades epileptogênicas (Kubová, Folbergrová e Mares, 1995; Folbergrová, 1997), atuando possivelmente via superativação de receptores glutamatérgicos, em especial os receptores *N*-metil-D-aspartato (NMDA) e metabotrópicos do grupo I (Lipton et al., 1997; Zieminska e Lazarewicz, 2006). Em adição, os efeitos neurodegenerativos da Hcy possivelmente estão associados à captação deste aminotiol por transportadores de membrana, promovendo acúmulo intracelular (Grieve, Butcher e Griffiths, 1992), e levando à morte neuronal via apoptose, que é precedida por dano ao DNA, ativação da poli (ADP-ribose) polimerase (PARP, EC 2.4.2.30) e das caspases, depleção energética e disfunção mitocondrial (Kruman et al., 2000). Ho e cols. (2002) desenvolveram um relevante estudo sobre os aspectos neurotóxicos da Hcy em culturas de neurônios. Os achados mostram que a Hcy promove o acúmulo citosólico de Ca^{2+}, a hiperfosforilação da Tau, induz o estresse oxidativo e a apoptose. Embora alguns desses efeitos possam ser prevenidos por antagonistas glutamatérgicos, reforçando o papel da excitotoxicidade nos efeitos mediados pela Hcy, o mecanismo de ação completo da Hcy permanece por ser esclarecido.

O estresse oxidativo tem sido um alvo recorrente de estudos envolvendo a Hcy, em parte porque a ativação de receptores NMDA pode ser seguida pela produção de espécies reativas (Kim e Pae, 1996; Ho et al., 2003; Jara-Prado et al., 2003), ou devido a geração de peróxido de hidrogênio, superóxido e hidroxil, durante a auto-oxidação da Hcy à homocistina e demais dissulfetos (Mudd, Levy e Kraus, 2001; Dayal et al., 2004; Faraci e Lentz, 2004). Nesse contexto, nosso grupo de pesquisa tem demonstrado a presença de estresse oxidativo em hipocampo e córtex cerebral induzido pela Hcy *in vitro*, evidenciado pela redução nas defesas antioxidantes e aumento na peroxidação lipídica (Streck et al., 2003b; Matté et al., 2004).

Por outro lado, poucos estudos têm contemplado a atuação da Hcy sobre o metabolismo energético. A depleção dos estoques energéticos (Kruman et al., 2000), e a inibição do metabolismo oxidativo (Streck et al., 2003a) podem estar associados à neurodegeneração causada pela Hcy. A Na^+,K^+-ATPase (EC 3.6.3.9) consome aproximadamente 50% do ATP produzido no cérebro a fim de manter a excitabilidade neuronal (Erecinska e Silver, 1994). Essa enzima integral de membrana é inibida pela Hcy em hipocampo (Streck et al., 2002a; Streck et al., 2002b; Wyse et al., 2002) e córtex parietal de ratos (Matté et al., 2004; Matté et al., 2006).

Os efeitos da Hcy sobre as células hepáticas têm atraído a atenção de um número menor de pesquisadores, resultando em um número restrito de dados sobre a toxicidade desse aminotiol no órgão que centraliza o seu metabolismo. Robert e cols. (2005) avaliaram o efeito da hiper-homocisteinemia sobre o fígado de camundongos CBS-deficientes, e demonstraram a presença de infiltrado inflamatório, ativação de células estreladas hepáticas e fibrose. Em concordância com esses dados, a hiper-homocisteinemia induz a produção de mediadores inflamatórios no fígado de ratos, que também podem ser detectados no plasma (Ji e Kaplowitz, 2004; Woo, Siow e O, 2008).

Considerando os efeitos da Hcy sobre o sistema vascular, uma revisão de McCully (2007) correlacionou o aparecimento de placas arterioscleróticas à hiper-homocisteinemia provocada pela deficiência na enzima CBS observada na HCU, mas também resultante de fatores genéticos, metabólicos, hormonais, nutricionais e tóxicos. A Hcy parece comprometer as células vasculares via ação pró-inflamatória, resultando nas modificações do tecido conjuntivo responsáveis pela formação das placas arterioscleróticas, caracterizadas por fibrose, calcificação e deposição de proteoglicanos. Além disso, foram observadas alterações pró-coagulantes, e o estímulo à oxidação e à fagocitose das partículas de lipoproteínas de baixa densidade (LDL).

4. Ácido fólico

O ácido fólico, ou vitamina B_9, é um derivado sintético dos folatos, estruturalmente caracterizados por uma molécula 2-amino-4-hidroxipteridina ligada por um carbono metileno ao *p*-aminobenzoilpoliglutamato (Figura 2). As espécies plasmáticas caracterizam-se pela presença de apenas uma molécula de glutamato, enquanto as intracelulares possuem de 5-8 moléculas de glutamato unidas através de ligações peptídicas γ-carboxila, em uma reação catalisada pela folilpoli-γ-glutamato sintetase (EC 6.3.2.17). Os poliglutamatos possuem uma afinidade aumentada às enzimas do ciclo dos folatos, permitindo o sequestro dessas espécies no meio intracelular e estimulando a canalização dos substratos nessa via (Suh, Herbig e Stover, 2001; Finglas et al., 2003).

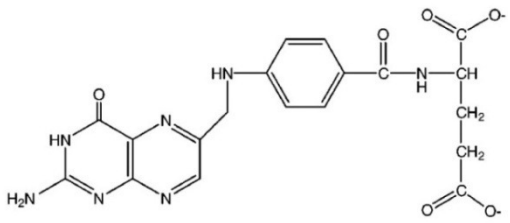

Figura 2. Estrutura molecular do ácido fólico (adaptado de Suh, Herbig e Stover, 2001).

O ácido fólico, com concentração hepática de aproximadamente 25-35 μM, é compartimentalizado na célula, onde aproximadamente 50% do conteúdo é citosólico e o restante encontra-se na mitocôndria. O ácido fólico participa do metabolismo de um carbono, onde é primeiramente convertido em diidrofolato e tetraidrofolato, em duas reações catalisadas pela enzima diidrofolato redutase (EC 1.5.1.3), e posteriormente são sintetizadas as coenzimas que atuam como doadores de grupos em três diferentes estados de oxidação, denominados metil, metileno e formil (Figura 3). O ácido fólico participa da síntese de bases nitrogenadas dos ácidos nucléicos, via síntese *de novo* de purinas e de timidilato. Além disso, exerce um papel fundamental no ciclo da Met. A

reação catalisada pela enzima MS, que é dependente da coenzima N^5-metiltetraidrofolato, transfere um grupo metil à Hcy (destacado na Figura 1), que posteriormente é doado aos aceptores de metil descritos no metabolismo da Hcy (Suh, Herbig e Stover, 2001; Finglas et al., 2003).

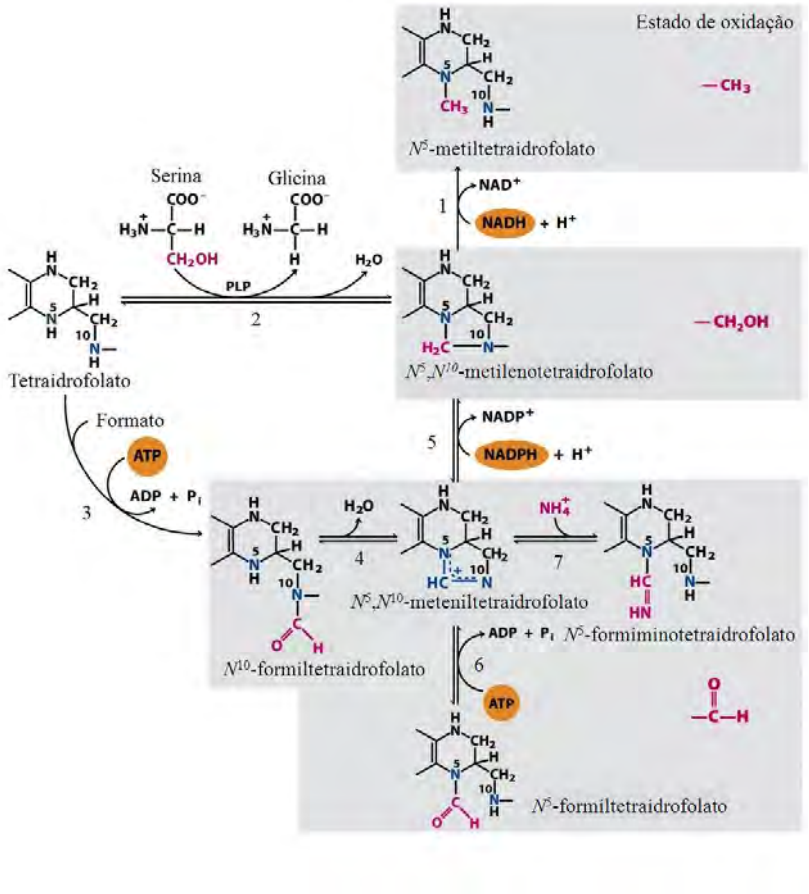

Figura 3. Ciclo dos folatos e coenzimas derivadas do ácido fólico (adaptado de Cox e Nelson, 2001). ADP, difosfato de adenosina; ATP, trifosfato de adenosina; PLP, piridoxal fosfato; NADH, nicotinamida adenina dinucleotídeo (forma reduzida); NADPH, nicotinamida adenina dinucleotídeo fosfato (forma reduzida). Enzimas: (1) N^5,N^{10}-metilenotetraidrofolato redutase (EC 1.5.1.20), (2) Serina hidroximetil transferase (EC 2.1.2.1), (3) N^{10}-formiltetraidrofolato sintetase (EC 6.3.4.3), (4) cicloidrolase (EC 3.5.4.9), (5) N^5,N^{10}-metilenotetraidrofolato desidrogenase (EC 1.5.1.5), (6) N^5,N^{10}-metilenotetraidrofolato sintetase (EC 6.3.3.2), (7) ciclodesaminase (EC 4.3.1.4)

Os níveis de folato em humanos podem ser determinados em soro (normal>3,5 ng/mL) ou eritrócitos (normal>140 ng/mL), sendo que no último as concentrações são representativas dos estoques corporais dessa vitamina. A determinação de folatos inclui o N^5-metiltetraidrofolato, produzido pela mucosa intestinal durante o processo de absorção, e a forma mais oxidada, o ácido fólico, quando a absorção de folatos da dieta excede a capacidade metabólica intestinal. As concentrações sanguíneas de folato estão

na ordem de nM, contrastando com as concentrações citosólicas na ordem de μM. Os altos níveis intracelulares de folato são obtidos através de um sistema de transporte presente na membrana celular, mediado por receptor de folato, carreadores de folatos reduzidos, e difusão passiva (Suh, Herbig e Stover, 2001). O sistema de transporte mediado por receptor também se encontra disponível na barreira hemato-encefálica (Wu e Pardridge, 1999). Por outro lado, a exportação de folatos só é possível após ação da enzima γ-glutamil hidrolase (EC 3.4.19.9), presente nos lisossomos, que atua hidrolisando as ligações peptídicas do poliglutamato, permitindo o transporte ATP-dependente para o meio extracelular (Suh, Herbig e Stover, 2001).

Essa vitamina do complexo B é encontrada em vegetais verdes, algumas frutas cítricas, fígado, derivados do leite e cereais. Sua labilidade ao cozimento, evidenciada por uma redução de 90% no conteúdo de folato, justifica a suplementação de farinhas com ácido fólico implementada nos Estados Unidos, Canadá, Chile, entre outros (Finglas et al., 2003). Embora essa suplementação dietética tenha apresentado uma redução nos casos de defeitos no fechamento do tubo neural, ainda não existem dados consistentes sobre as doses mais eficazes para a ampla prevenção dessas doenças. A suplementação com ácido fólico não apresenta indícios de toxicidade, apesar de alguns casos de deficiência de cobalamina serem mascarados (Eichholzer, Tönz e Zimmermann, 2006).

O ácido fólico tem sido utilizado clinicamente, em doses de 0,4-5 mg/dia, em uma série de situações, tais como a prevenção de defeitos no fechamento do tubo neural (Finglas et al., 2003; Blom et al., 2006), em pacientes epiléticos em tratamento, desde que alguns fármacos reduzem substancialmente os níveis dessa vitamina (Morrell, 2002), bem como na prevenção de doenças cardiovasculares (McCully, 2007), neurodegenerativas (Mattson, Kruman e Duan, 2002; Mattson e Shea, 2003) e

psiquiáticas (Bottiglieri, 2005; Miller, 2008). Em muitas dessas situações patológicas observa-se o envolvimento da hiper-homocisteinemia, o que justifica a utilização de ácido fólico baseada na participação do N^5-metiltetraidrofolato no metabolismo da Hcy (Eskes, 1998; Mattson, Kruman e Duan, 2002; Mattson e Shea, 2003; Bottiglieri, 2005; Blom et al., 2006; McCully, 2007; Miller, 2008). Apesar de alguns estudos, incluindo meta-análises, demonstrarem a inefetividade do tratamento com ácido fólico em doenças cardiovasculares (Bazzano et al., 2006; Collaboration, 2006; Clarke et al., 2007) e neurodegenerativas (Luchsinger e Mayeux, 2004; Clarke et al., 2007); os dados descritos acima permitem sugerir que o ácido fólico possui um papel promissor no tratamento e prevenção de doenças que envolvem o SNC e vascular, entretanto mais estudos são necessários a fim de definir as doses e o tempo de tratamentos mais eficazes.

Mais recentemente, um novo papel tem sido delineado para o ácido fólico, pertinente a sua ação antioxidante na prevenção dos efeitos cardiovasculares da Hcy (Brattström e Wilcken, 2000; Doshi et al., 2001). Nesse contexto, Joshi e cols. (2001) descrevem a ação como sequestradores de espécies reativas em pH fisiológico, tais como radical tiólico, peroxil e hidroxil, bem como prevenindo a peroxidação lipídica microssomal. Au-Yeung e cols. (2006) demonstraram atividade antioxidante e anti-inflamatória do ácido fólico sobre macrófagos ativados por Hcy, via inibição da produção de superóxido pela enzima nicotinamida adenina dinucleotídeo fosfato oxidase (NADPH oxidase, EC 1.6.3.1). Em concordância, a suplementação de pacientes hiper-homocisteinêmicos (>15 μM) com 5 mg diários de ácido fólico por um período de dois meses reduziu os níveis de Hcy, associado a uma melhora nos níveis antioxidante eritrocitário e à redução na peroxidação lipídica plasmática (Racek et al., 2005).

5. Estresse oxidativo

O estresse oxidativo é caracterizado por um distúrbio no equilíbrio entre pró-oxidantes e antioxidantes, resultando em dano celular. Esse desequilíbrio pode estar associado a uma redução no conteúdo celular de antioxidantes, em decorrência da utilização excessiva desse sistema, ou da produção e ingestão insuficientes. Por outro lado, o estresse oxidativo também pode ser consequência da produção excessiva de espécies reativas, que podem ser obtidas por ingestão de certas toxinas, ou produzidas pelo metabolismo celular em situações fisiológicas e patológicas (Halliwell e Whiteman, 2004).

Antioxidantes são conceituados como substâncias que quando presentes em baixas concentrações, em comparação a substratos oxidáveis, previnem significantemente a oxidação do substrato, que pode ser qualquer biomolécula (Halliwell e Whiteman, 2004). Os antioxidantes são divididos em duas classes, enzimáticos e não-enzimáticos. Dentre os enzimáticos, destacamos o papel da superóxido dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutationa peroxidase (GPx, EC 1.11.1.9) e glutationa redutase (GR, EC 1.8.1.7), que atuam coordenadamente na detoxificação das espécies reativas de oxigênio (ERO) (Figura 4). Os antioxidantes não-enzimáticos são um grupo heterogêneo de moléculas, que podem ser produzidas endogenamente ou obtidas da dieta, tais como as vitaminas A, C, E, riboflavina e tiamina; os polifenóis, os carotenóides, e os compostos de baixo peso molecular, que incluem bilirrubina, α-cetoácidos, melatonina, urato, ácido lipóico, estrógenos e GSH; assim como as proteínas ligadoras de ferro e cobre, transferrina e ceruloplasmina, respectivamente (Halliwell, 2001; Salvador e Henriques, 2004). Recentemente, o ácido fólico também tem sido descrito como um potencial antioxidante

(Joshi et al., 2001; Racek et al., 2005; Au-Yeung et al., 2006), com promissora utilização na clinica.

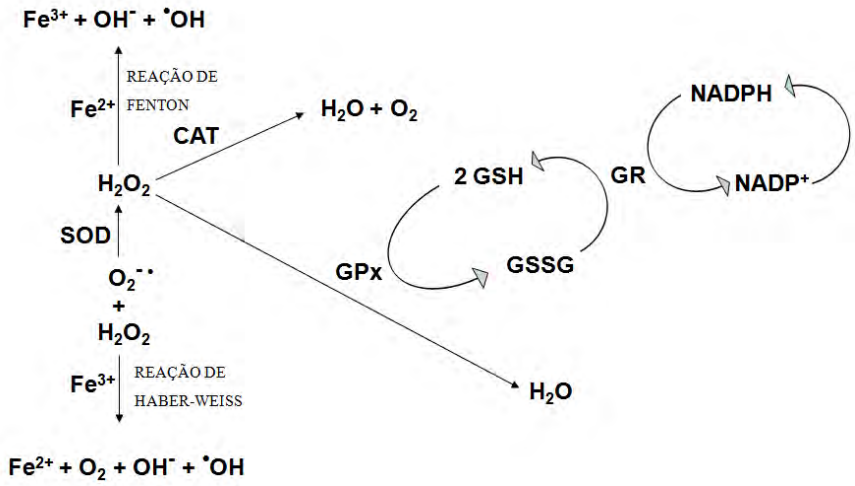

Figura 4. Sistema enzimático que atua sobre as espécies reativas de oxigênio (adaptado de Salvador e Henriques, 2004). CAT, catalase; GPx, glutationa peroxidase; GR, glutationa redutase; GSH, glutationa (forma reduzida); GSSG, glutationa (forma oxidada); SOD, superóxido dismutase

O dano oxidativo celular pode ter como consequências a adaptação celular, caracterizada pelo aumento na produção de defesas antioxidantes; ou o dano às biomoléculas, tais como lipídios, proteínas, DNA e carboidratos; e em situações limites, a morte celular via necrose ou apoptose. Biologicamente, as espécies reativas mais importantes como mediadores de dano oxidativo são as ERO, tais como o peróxido de hidrogênio (H_2O_2), e os radicais superóxido ($O_2^{\bullet -}$), hidroxil ($OH^{\bullet}$) e peroxil ($RO_2^{\bullet}$); embora as espécies reativas de nitrogênio, tais como o óxido nítrico ($NO^{\bullet}$) e o dióxido de nitrogênio ($NO_2^{\bullet}$), e os radicais de enxofre ou tiólicos também apresentem papéis relevantes na indução de estresse oxidativo (Halliwell e Whiteman, 2004).

As ERO são produzidas predominantemente na mitocôndria, onde 2-5% do oxigênio sofre redução monoeletrônica, principalmente nos complexos I, II e III da cadeia transportadora de elétrons, gerando superóxido (Halliwell e Whiteman, 2004; Kowaltowski et al., 2009). Além disso, esse radical livre também é produzido via auto-oxidação de uma série de moléculas, tais como a Hcy (Mudd, Levy e Kraus, 2001) e a

dopamina (Fahn e Cohen, 1992). Esse radical livre é convertido a peróxido de hidrogênio pela enzima SOD, que possui isoenzimas diferentes na matriz mitocondrial (Mn-SOD) e no espaço intermembranas e citosol (Cu,Zn-SOD). O peróxido de hidrogênio é relativamente mais estável e pode atravessar as membranas biológicas, sendo convertido à água através das reações catalisadas pela CAT, GPx ou tioredoxina peroxidase (EC 1.11.1.15), que possuem localizações celulares diferenciadas e complementares. O peróxido de hidrogênio também pode ser convertido, através das reações de Fenton e Haber-Weiss, em radical hidroxil, que possui o maior potencial de dano às biomoléculas (Figura 4). Fisiologicamente algumas ERO têm papéis importantes no metabolismo e na sinalização celular, tais como o óxido nítrico, um neurotransmissor, e o peróxido de hidrogênio, que atua como segundo mensageiro na ativação do fator nuclear kappa B (NFκB), bem como no processo inflamatório (Halliwell e Whiteman, 2004).

Apesar de algumas espécies reativas, tais como o óxido nítrico, o superóxido e o peróxido de hidrogênio, apresentarem um papel relevante na potenciação de longa duração (LTP); o estresse oxidativo parece afetar a memória no envelhecimento (Serrano e Klann, 2004), além de estar associado a uma série de patologias que atingem o SNC, tais como as doenças neurodegenerativas, esclerose lateral amiotrófica, síndrome de Down e desordens psiquiátricas (Halliwell, 2001; Halliwell, 2006; Andreazza et al., 2009; Rezin et al., 2009).

Nesse sentido, sabe-se que o cérebro possui uma série de fatores que colaboram para o desenvolvimento do dano oxidativo, desde que esse órgão consome aproximadamente 20% do oxigênio disponível ao organismo. O metabolismo cerebral é predominantemente oxidativo, visando a síntese de ATP, que é utilizado principalmente na manutenção do gradiente iônico celular pela Na^+,K^+-ATPase e na estocagem de

neurotransmissores. A suscetibilidade do cérebro também se deve aos neurotransmissores presentes nesse tecido, a dopamina, a serotonina e a noradrenalina geram superóxido via auto-oxidação; enquanto o glutamato, que é o principal neurotransmissor presente no SNC, participa de processos excitotóxicos, onde o influxo excessivo de Ca^{2+} induz a geração de espécies reativas. O cérebro é rico em ferro, que pode catalisar a formação de radical hidroxil, a partir de peróxido de hidrogênio (Figura 4). Os ácidos graxos poliinsaturados presentes em alta concentração nas membranas celulares cerebrais, especialmente ácido docosaexaenóico (22:6 $\Delta^{4,7,10,13,16,19}$), são extremamente sensíveis à peroxidação. Por outro lado, as defesas antioxidantes cerebrais são modestas, quando comparadas a outros órgãos, principalmente em referência a atividade da CAT (Halliwell, 2001; Halliwell, 2006).

6. Metabolismo energético

A mitocôndria possui um papel central na manutenção da vida celular, desde que é o centro de produção de ATP, via fosforilação oxidativa. Nesse contexto, o acetil-CoA é o produto metabólico de uma série de macromoléculas obtidas da dieta, tais como ácidos graxos, aminoácidos e glicose, concentrando os esqueletos carbônicos desses compostos na matriz mitocondrial para a sequência no metabolismo oxidativo. As coenzimas reduzidas, nicotinamida adenina dinucleotídeo (NADH) e flavina adenina dinucleotídeo ($FADH_2$), produzidas nessas reações catabólicas serão re-oxidados na cadeia transportadora de elétrons. Quatro complexos enzimáticos são responsáveis pela transferência de elétrons desde as coenzimas reduzidas até o aceptor final, o oxigênio, num mecanismo dependente do potencial padrão de óxido-redução de cada par redox (Figura 5). Os elétrons provenientes do NADH são entregues ao complexo I, também denominado NADH desidrogenase (EC 1.6.99.3), que os transfere à ubiquinona, uma

coenzima móvel que também é responsável pelo transporte dos elétrons provenientes do $FADH_2$, o grupo prostético da succinato desidrogenase (SDH, EC 1.3.99.1), uma enzima do ciclo de Krebs ligada à membrana mitocondrial interna, onde faz parte do complexo II (EC 1.3.5.1). Os elétrons da ubiquinona são transferidos ao complexo III, também denominado ubiquinona:citocromo *c* oxidoredutase (EC 1.10.2.2), e posteriormente ao citocromo *c*, uma molécula móvel, que participa da transferência eletrônica ao complexo IV, a citocromo *c* oxidase (COX, EC 1.9.3.1), mas também está diretamente envolvido no mecanismo da apoptose. Finalmente os elétrons são transferidos ao oxigênio produzindo água, numa reação catalisada pela COX. Concomitante à transferência eletrônica, do sítio mais eletronegativo ao sítio mais eletropositivo, ocorre a liberação de energia, que é conservada através de um gradiente de prótons, os quais são bombeados da matriz mitocondrial para o espaço intermembranas pelos complexos I, III e IV da cadeia respiratória. Esse mecanismo é a base do modelo quimiosmótico, descrito por Peter Mitchell em 1961, que explica o acoplamento entre a formação do gradiente eletroquímico, gerador da força próton-motriz, que é utilizado pelo complexo F_oF_1-ATPase (EC 3.6.3.14) para a síntese de ATP, um processo denominado fosforilação oxidativa (Cox e Nelson, 2001).

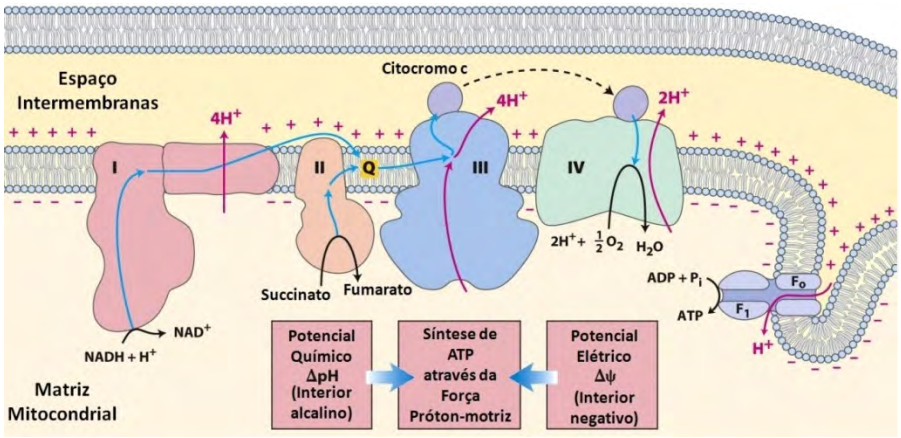

Figura 5. Cadeia transportadora de elétrons (adaptado de Cox e Nelson, 2001). ADP, difosfato de adenosina; NADH, nicotinamida adenina dinucleotídeo (forma reduzida); Q, ubiquinona

Os processos descritos até aqui para a respiração celular também se aplicam ao SNC, entretanto assim como os tipos celulares cerebrais apresentam uma divisão de funções, também parecem diferir em relação ao metabolismo energético, atuando de forma conectada através da lançadeira de lactato astrócito-neurônio, descrita por Luc Pellerin e Pierre Magistretti. Estimulando o metabolismo energético via glutamato, esses pesquisadores mostraram que a glicose sanguínea, o principal substrato oxidável do SNC, é captada principalmente por astrócitos, via transportador de glicose (GLUT) 1, enquanto os neurônios captam uma porcentagem menor de glicose via GLUT-3. Nos astrócitos, a glicose é convertida em piruvato, pela via glicolítica, e esse à lactato pela lactato desidrogenase (LDH, EC 1.1.1.27) 5, a fim de re-oxidar o NADH produzido pela via glicolítica, desde que as mitocôndrias astrocitárias não apresentam a lançadeira malato-aspartato plenamente funcionante. O lactato é transportado para o meio extracelular e captado por neurônios, onde é re-convertido em piruvato, através da enzima LDH-1, e segue o metabolismo oxidativo mitocondrial para produção de ATP (Pellerin e Magistretti, 2004; Pellerin, 2008).

A creatina quinase (CK, EC 2.7.3.2) é uma enzima importante para o metabolismo energético de células com alta demanda energética, como é o caso do SNC e tecido muscular, desde que atua como um tampão energético e um transportador de compostos de alta energia do sítio de produção, a mitocôndria, até o local de consumo, o citosol. A CK, que apresenta isoenzimas mitocondriais e citosólicas, catalisa a transferência reversível de grupos fosfato de alta energia entre o ATP e a creatina, produzindo ADP e fosfocreatina, numa reação dependente das necessidades energéticas momentâneas da célula. Nesse contexto, a CK parece estar acoplada a Na^+,K^+-ATPase, que utiliza aproximadamente metade do ATP produzido no SNC (Wallimann e Hemmer, 1994).

A Na^+,K^+-ATPase é uma enzima integral de membrana, que mantém o gradiente iônico celular através do co-transporte de três íons Na^+ para o meio extracelular e dois íons K^+ para o meio intracelular, conservando o gradiente de Na^+, indispensável à captação de glutamato e a geração do potencial de membrana, a fim de manter a excitabilidade e o volume neuronal (Kaplan, 2002; Devlin, 2003). Essa enzima possui ampla distribuição tecidual, apesar de ser encontrada em maior concentração em células epiteliais e neurais (Hansen e Clausen, 1988). O SNC apresenta uma elevação gradativa da atividade da Na^+,K^+-ATPase após o nascimento, que se co-localiza temporalmente com as alterações durante a sinaptogênese ativa, sendo que uma maior atividade desta enzima ocorre na região sináptica (Erecinska, Cherian e Silver, 2004). Nesse contexto, diversos estudos têm demonstrado o papel dessa enzima nos mecanismos de memória e LTP (Brunelli et al., 1997; Glushchenko e Izvarina, 1997).

Alterações mitocondriais têm sido relatadas em uma série de doenças psiquiátricas, tais como desordem bipolar, depressão e esquizofrenia (Rezin et al., 2009), e doenças neurodegenerativas (Beal, 2000; Sullivan e Brown, 2005; Zeviani e Carelli, 2007). Acredita-se que a geração de ERO esteja profundamente ligada à disfunção mitocondrial (Cadenas e Davies, 2000; Nicholls e Budd, 2000; Nicholls, 2008), que está associada à peroxidação lipídica e alteração na atividade dos complexos da cadeia transportadora de elétrons (Muralikrishna Adibhatla e Hatcher, 2006).

7. Sistema glutamatérgico

O glutamato é o principal neurotransmissor excitatório do SNC, sendo utilizado por aproximadamente dois terços das sinapses do neocórtex e do hipocampo (Fonnum, 1984). A maior parte do glutamato é sintetizada no neurônio pré-sináptico a partir da glutamina derivada das células gliais, e posteriormente estocado em vesículas (Figura 6). A exocitose dessas vesículas é Ca^{2+}-dependente e promove a liberação de altas

concentrações de glutamato na fenda sináptica, onde atuará nos receptores glutamatérgicos. Os receptores podem ser divididos em dois grandes grupos: os ionotrópicos, que contém um canal iônico na sua estrutura, compreendendo os receptores ácido α-amino-3-hidróxi-5-metil-4-isoxazol-propiônico (AMPA), cainato e NMDA; e os receptores metabotrópicos, que estão acoplados à proteína G, e são subdivididos em três grupos ($mGluR_{1-8}$). A finalização da ação glutamatérgica ocorre via captação do glutamato, geralmente Na^+-dependente, realizada por transportadores especializados presentes nas membranas gliais e neuronais (Danbolt, 2001; Squire et al., 2003; Magistretti, 2006). Como descrito acima, a Na^+,K^+-ATPase mantém o gradiente de Na^+ necessário à captação de glutamato e *clearance* da fenda sináptica. Os transportadores de glutamato descritos até o momento são cinco: o transportador glial de glutamato (GLAST)/transportador de aminoácidos excitatórios (EAAT) 1 e o transportador glutamato-aspartato dependente de Na^+ (GLT-1)/EAAT2 estão presentes predominantemente nas células gliais, enquanto o carreador de aminoácidos excitatórios 1 (EAAC1)/EAAT3 é encontrado principalmente na membrana neuronal pós-sináptica, o EAAT4 foi descrito principalmente em células de Purkinge no cerebelo, e o EAAT5 presente nas células da retina. GLAST e GLT-1 são quantitativamente os principais transportadores de glutamato no SNC, contribuindo com aproximadamente 90% da captação de glutamato, impedindo a excitotoxicidade mediada pela permanência desse neurotransmissor excitatório na fenda sináptica (Danbolt, 2001; Maragakis e Rothstein, 2004).

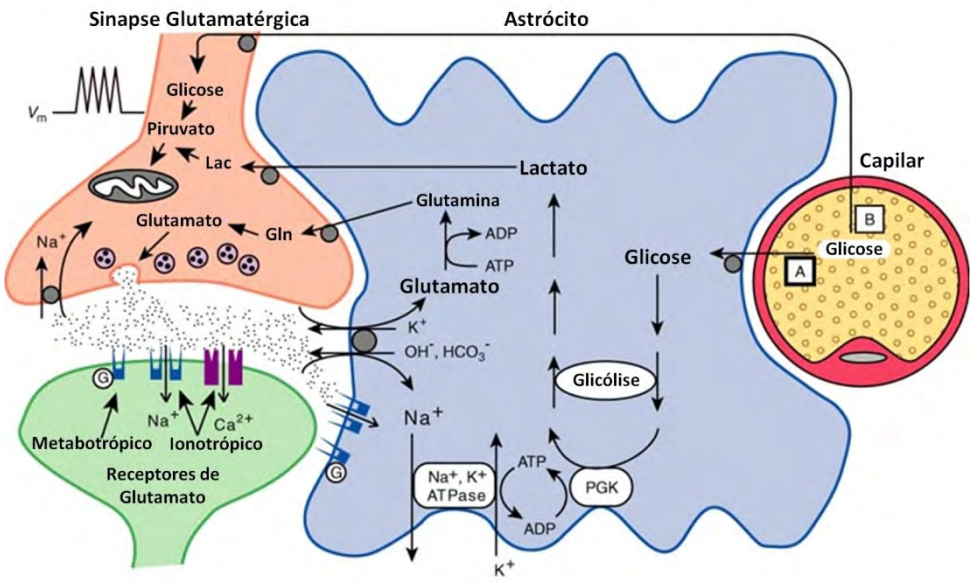

Figura 6. Sinapse glutamatérgica (adaptado de Squire et al., 2003, Fundamental Neuroscience). ADP, difosfato de adenosina; ATP, trifosfato de adenosina; G, proteína G; Gln, glutamina; Lac, lactato; PGK, fosfoglicerato quinase (EC 2.7.2.3); V_m, potencial de membrana

O glutamato possui um papel dual no SNC, assim como é essencial nos processos de aprendizado e memória, também pode levar a morte celular. Nesse contexto, a excitotoxicidade glutamatérgica tem início no aumento das concentrações citoplasmáticas de Ca^{2+} através de: (1) ativação dos receptores ionotrópicos, inicialmente os receptores do tipo AMPA permitem o influxo de Ca^{2+} e Na^{+}, despolarizando a célula pós-sináptica, o que retira o bloqueio aos receptores NMDA, promovido pelo íon Mg^{2+}, levando ao influxo maciço de Ca^{2+}, (2) ativação de receptores metabotrópicos, resultando na mobilização dos estoques intracelulares de Ca^{2+}, via produção de inositol trifosfato (IP_3), e (3) abertura dos canais de Ca^{2+} dependentes de voltagem, resultado da despolarização mediada pelos receptores AMPA. A mitocôndria capta o excesso de Ca^{2+} do citosol, o que pode induzir a produção de ERO, e consequentemente o estresse oxidativo, além de inibir a produção de ATP pela fosforilação oxidativa. O Ca^{2+} também é responsável pela ativação de calpaínas, caspases e proteínas pró-apoptóticas, desencadeando a morte celular via apoptose (Mattson, 2003).

8. Memória

A memória é um processo complexo e que contempla ao menos as fases de aquisição, consolidação e evocação (Izquierdo, 2002). Recentemente uma nova fase denominada persistência da memória têm sido caracterizada bioquimicamente (Bekinschtein et al., 2007).

Diversas formas de classificação têm sido utilizadas para a memória. De acordo com o conteúdo, as memórias podem ser identificadas como declarativa e não-declarativa, que também são denominadas memória de fatos e habilidades, ou explícita e implícita, respectivamente. Utilizando o fator tempo, as memórias são classificadas como memória de curta duração (STM) e memória de longa duração (LTM). Considerando a função, abrangemos as memórias de trabalho e de referência. De acordo com a motivação, obtemos as memórias de recompensa e aversivas. O lobo temporal medial, que inclui o hipocampo, a amígdala, e o córtex vizinho, é a região do encéfalo que se acredita tenha um papel central no processamento da memória declarativa; enquanto a amígdala, o cerebelo e o estriado parecem estar associados ao processamento da memória não-declarativa (Squire e Kandel, 2003).

A memória não-declarativa possui um caráter robusto, sendo que uma vez estabelecida pode permanecer pelo restante da vida de um indivíduo, dificilmente é extinta, além de não possuir influência emocional. Por outro lado, a memória declarativa apresenta uma dependência de estado, e pode ser expressa como STM, que possui duração de minutos até 1-3 horas, e LTM, que pode persistir por horas, dias e até anos. As memórias de curta e longa duração possuem diferentes mecanismos de consolidação, e não são processos sequenciais de uma mesma memória; apesar de utilizarem as mesmas estruturas cerebrais, os mecanismos neuroquímicos envolvidos são bastante distintos (Izquierdo, 2002; Izquierdo et al., 2002).

Acredita-se que a memória seja estocada nas sinapses, e que a plasticidade sináptica seja o mecanismo celular para a memória e o aprendizado (Lu, Christian e Lu, 2008). Nesse contexto, a consolidação da STM não envolve a síntese de novas proteínas, parecendo estar mais associada à ativação de receptores de membrana pós-sinápticos, tais como os receptores AMPA e NMDA, em sinapses glutamatérgicas, resultando no influxo de Ca^{2+} e produção de mensageiros retrógrados, que retroalimentam as células pré-sinápticas a fim de aumentar a neurotransmissão (Squire e Kandel, 2003). Izquierdo e cols. (2002) revisaram os mecanismos envolvidos na consolidação da STM, e reportam a participação de receptores glutamatérgicos, colinérgicos muscarínicos, β-adrenérgicos, serotoninérgicos (5-HT1A) e dopaminérgicos D1; bem como de uma série de enzimas, tais como a adenilato ciclase (EC 4.6.1.1), proteína quinase A (PKA, EC 2.7.11.11), fosfatidil-inositol 3 quinase (PI3K, EC 2.7.1.137), tirosina quinases, guanilato ciclase (EC 4.6.1.2), proteína quinase G (PKG, EC 2.7.11.12), proteína quinase $C_{\alpha/\beta II}$ (PKC, EC 2.7.11.1) e da proteína quinase ativada por mitógeno (MAPK, EC 2.7.11.24) em diferentes estruturas do SNC.

Por outro lado, a consolidação da LTM envolve a expressão gênica e a síntese de proteínas, e parece ser mediada pelos mesmos mecanismos participantes da LTP induzida *in vitro*. Em linhas gerais, a LTP depende da ativação repetida de alta frequência do neurônio pré-sináptico no hipocampo, promovendo a liberação de neurotransmissores, como o glutamato. Na sequência são ativados os receptores glutamatérgicos pós-sinápticos (AMPA e posteriormente o NMDA), promovendo o influxo de Ca^{2+}, que ativa uma série de quinases, culminando no acionamento da transcrição gênica, síntese de novos receptores de membrana e no aumento da neurotransmissão (Squire e Kandel, 2003). Os efeitos pré-sinápticos indicam a participação de mensageiros retrógrados, tais como o óxido nítrico (Squire e Kandel,

2003) e o fator neurotrófico derivado do encéfalo (BDNF) (Bramham e Messaoudi, 2005; Soulé, Messaoudi e Bramham, 2006). Associadas a essas transformações moleculares na fase precoce da LTP, modificações estruturais também têm sido descritas nas sinapses na fase tardia da LTP, caracterizadas pelo crescimento de novos sítios sinápticos (Squire e Kandel, 2003). Os mecanismos moleculares que participam da consolidação da LTM são muito semelhantes aos da STM, envolvendo receptores glutamatérgicos, colinérgicos muscarínicos, β-noradrenérgicos, dopaminérgicos D_1, e serotoninérgicos (5-HT1A), assim como as enzimas adenilato ciclase, PKA, PI3K, tirosina quinases, guanilato ciclase, PKG, PKC e a via da Erk em diversas estruturas cerebrais (Izquierdo et al., 2002).

O BDNF possui seu metabolismo atrelado à ativação neuronal, garantindo, assim, a especificidade de ação na sinapse ativa; onde parece modular a transmissão sináptica e a sinaptogênese (Thomas e Davies, 2005; Bekinschtein et al., 2008). Essa neurotrofina é sintetizada como uma pró-proteína no retículo endoplasmático, e pode ser clivada intra ou extracelularmente. Após ser vesiculada, num processo dependente da carboxipeptidase E (EC 3.4.16), é secretada por uma via regulada pela atividade sináptica. As neurotrofinas exercem suas ações celulares através da ligação em duas classes de receptores, a família tirosina quinase (Trk) e o $p75^{NTR}$. O BDNF encontra-se co-localizado, pré e pós-sinapticamente, com os receptores TrkB em sinapses glutamatérgicas, em hipocampo e córtex cerebral (Bekinschtein et al., 2008; Carvalho et al., 2008). A ligação a esses receptores pode levar a ativação das vias de sinalização da PI3K, da fosfolipase C γ (PLCγ, EC 3.1.4.3) e da Erk 1/2. O BDNF tem sido relacionado à consolidação das memórias de curta e de longa duração (Alonso et al., 2002; Alonso et al., 2005; Bramham e Messaoudi, 2005; Soulé, Messaoudi e Bramham, 2006; Bekinschtein et al., 2008). Nesse contexto, essa neurotrofina promove a

consolidação sináptica através da facilitação da liberação de glutamato, e da modulação da fosforilação e translocação de receptores AMPA e NMDA para a membrana. O BDNF também regula a transcrição via fosforilação do elemento de ligação responsivo ao AMPc (CREB), modulando a síntese de proteínas dendríticas relacionadas à plasticidade sináptica; além de aumentar o número e o tamanho das espinhas dendríticas (Bramham e Messaoudi, 2005; Soulé, Messaoudi e Bramham, 2006; Bekinschtein et al., 2008; Tyler et al., 2008). O BDNF tem sido associado à consolidação de diferentes tipos de memória, diferentes estágios pós-aprendizado, e diferentes tarefas comportamentais em ratos, tais como esquiva inibitória (Alonso et al., 2002; Alonso et al., 2005; Bekinschtein et al., 2008) e labirinto aquático de Morris (McGauran et al., 2008; Tyler et al., 2008).

Diversas tarefas podem ser empregadas a fim de avaliar diferentes estágios e tipos de memórias. Em função dos objetivos dessa tese, foram utilizadas as tarefas de esquiva inibitória, labirinto aquático de Morris e campo aberto. A tarefa de esquiva inibitória apresenta uma característica aversiva, desde que o comportamento exploratório natural do rato é punido com um choque, o que também é um estímulo para o aprendizado da tarefa. Esse paradigma não apresenta uma clara classificação quanto à memória avaliada, incluindo a memória declarativa, que compreende um componente associativo (ao contexto do aparato); e o condicionamento operante (devido ao choque), condizente com a memória não-declarativa. A tarefa de esquiva inibitória é dependente do hipocampo, da amígdala, e dos córtices entorrinal e parietal, que parecem atuar nessa ordem durante a consolidação da memória (Izquierdo et al., 1997). O labirinto aquático de Morris é um paradigma que avalia a memória declarativa espacial dos ratos, que é dependente do hipocampo, mas também apresenta uma participação importante dos córtices parietal e pré-frontal (Morris, 1984; D'Hooge e De Deyn, 2001; Saito e

Watanabe, 2006; Save e Poucet, 2009). A tarefa de campo aberto, caracterizada como não-aversiva e não-associativa, é utilizada como controle da atividade motora do animal (Xavier, 1999).

II. OBJETIVOS

Objetivo geral

A presente tese de doutorado apresentou como objetivo principal avaliar o efeito da hiper-homocisteinemia sobre parâmetros comportamentais e bioquímicos em cérebro, sangue e fígado de ratos. Foram analisados parâmetros de estresse oxidativo, de metabolismo energético e relativos à captação de glutamato. O papel neuroprotetor do ácido fólico também foi avaliado.

Objetivos específicos

Os objetivos específicos referem-se aos seis capítulos, na forma de artigos científicos, em que foi subdivida a presente tese de doutorado:

Capítulo I

✓ Analisar o efeito da administração crônica de ácido fólico sobre as alterações nos parâmetros de estresse oxidativo, denominados potencial antioxidante total não-enzimático (TRAP), atividade das enzimas antioxidantes (SOD, CAT e GPx), assim como sobre o dano ao DNA em córtex parietal e sangue de ratos submetidos à administração crônica de Hcy.

Capítulo II

✓ Avaliar o efeito da hiper-homocisteinemia crônica sobre alguns parâmetros de estresse oxidativo, denominados TRAP, reatividade antioxidante total (TAR), atividade da CAT, parâmetros de peroxidação lipídica (quimioluminescência induzida e substâncias reativas ao ácido tiobarbitúrico – TBARS) e medida do conteúdo de tióis totais, em fígado de ratos.

✓ Determinar a atividade das aminotransferases em fígado e plasma de animais submetidos ao modelo de hiper-homocisteinemia.

✓ Realizar análise histológica do tecido hepático, avaliando a morfologia, a presença de esteatose, infiltrado inflamatório, fibrose e conteúdo de glicogênio/glicoproteínas em ratos tratados cronicamente com Hcy.

Capítulo III

✓ Analisar o efeito das administrações aguda e crônica de Hcy sobre as atividades da CK, da SDH e dos complexos II e IV da cadeia respiratória em córtex parietal de ratos. Também foi investigado um possível efeito antioxidante do tratamento com ácido fólico sobre as alterações causadas pela Hcy.

Capítulo IV

✓ Investigar o efeito das administrações aguda e crônica de Hcy sobre a captação de glutamato e o imunoconteúdo dos transportadores gliais desse aminoácido (GLAST e GLT-1) em córtex parietal de ratos, durante o período de desenvolvimento do SNC de ratos.

Capítulo V

✓ Avaliar o efeito da administração crônica de ácido fólico sobre as alterações na memória espacial, medida no labirinto aquático de Morris, em ratos submetidos ao tratamento crônico com Hcy.

✓ Analisar o efeito do tratamento com ácido fólico sobre as alterações na atividade da Na^+,K^+-ATPase e na peroxidação lipídica em córtex parietal, bem como nos níveis plasmáticos e cerebrais de Hcy em ratos hiper-homocisteinêmicos.

Capítulo VI

✓ Investigar o efeito do pré-tratamento com ácido fólico sobre o prejuízo na consolidação da memória (STM e LTM), avaliada na tarefa de esquiva inibitória, e no imunoconteúdo cerebral de BDNF em ratos submetidos à administração aguda de Hcy.

PARTE II

III. RESULTADOS

Capítulo I

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[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Departamento de Análises Clínicas e Toxicológicas, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c] Instituto de Biotecnologia, Universidade de Caxias do Sul, Caxias do Sul, RS, Brazil

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[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Departamento de Análises Clínicas e Toxicológicas, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil
[c] Instituto de Biotecnologia, Universidade de Caxias do Sul, Caxias do Sul, RS, Brazil

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ABSTRACT

We have previously demonstrated that acute hyperhomocysteinemia induces oxidative stress in rat brain. In the present study, we initially investigated the effect of chronic hyperhomocysteinemia on some parameters of oxidative damage, namely total radical-trapping antioxidant potential and activities of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase), as well as on DNA damage in parietal cortex and blood of rats. We also evaluated the effect of folic acid on biochemical alterations elicited by hyperhomocysteinemia. Wistar rats received daily subcutaneous injection of Hcy (0.3–0.6 μmol/g body weight), and/or folic acid (0.011 μmol/g body weight) from their 6th to their 28th day of life. Twelve hours after the last injection the rats were sacrificed, parietal cortex and total blood was collected. Results showed that chronic homocysteine administration increased DNA damage, evaluated by comet assay, and disrupted antioxidant defenses (enzymatic and non-enzymatic) in parietal cortex and blood/plasma. Folic acid concurrent administration prevented homocysteine effects, possibly by its antioxidant and DNA stability maintenance properties. If confirmed in human beings, our results could propose that the supplementation of folic acid can be used as an adjuvant therapy in disorders that accumulate homocysteine.

1. Introduction

Tissue levels of homocysteine (Hcy) are increased in homocystinuria, an inborn error of metabolism characterized biochemically by cystathionine β-synthase (CBS, EC 4.2.1.22) deficiency. Clinically, patients present mental retardation, seizures and vascular complications (Mudd et al., 2001). Furthermore, moderate hyperhomocysteinemia also occur in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Diaz-Arrastia, 2000; Mattson et al., 2002; Mattson and Haberman, 2003; Sachdev, 2004; Regland, 2005), and has been identified as a risk factor for cancer (Wu and Wu, 2002) and vascular problems, such as stroke, cerebrovascular diseases and atherosclerosis (Welch and Loscalzo, 1998; Clarke et al., 1999; Eikelboom et al., 1999; Diaz-Arrastia, 2000; Ullegaddi et al., 2006).

The underlying mechanism by which Hcy exert its neurotoxic effects remain unexplained, however, some hypotheses have been described. It has been showed that Hcy induces oxidative stress by activation of glutamatergic receptors, with consequent reactive species generation (Kim and Pae, 1996; Zhang et al., 1998; Ho et al., 2003; Jara-Prado et al., 2003; Boldyrev et al., 2005; Zieminska and Lazarewicz, 2006), or by autoxidation to homocystine and other disulphides releasing $O_2^{\bullet -}$ and H_2O_2 (Kim and Pae, 1996; Ho et al., 2002; Dayal et al., 2004; Faraci and Lentz, 2004). We previously showed that Hcy induces oxidative stress in brain of rats, reducing antioxidant defenses and increasing lipid peroxidation (Wyse et al., 2002; Streck et al., 2003; Matté et al., 2004, 2007).

Oxidative stress appeared to possess an important role in neurodegenerative diseases and vascular complications (Hazell, 2007; Zhu et al., 2007; Shi and Liu, 2007; Halliwell, 2006). This condition can be defined as a serious imbalance between production of reactive species and antioxidant defenses, and could result from diminished levels of antioxidants and/or increased production of reactive species (Halliwell, 2001; Halliwell and Whiteman, 2004; Halliwell and Gutteridge, 2007). The brain is

* Corresponding author at: Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5573; fax: +55 51 3308 5535.
E-mail address: wyse@ufrgs.br (Angela T.S. Wyse).

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particularly vulnerable to oxidative stress, mainly by its high oxygen consumption, extensive use of glutamate as a neurotransmitter, high iron and polyunsaturated fatty-acid side-chains contents, low antioxidant levels compared to other organs, and by the presence of microglial cells, which can produce reactive oxygen species (ROS) (Halliwell and Gutteridge, 2007).

Folic acid exerts an important role over Hcy catabolism, by methyl group donation in remethylation pathway to methionine (Finkelstein, 1998; Brosnan et al., 2004). Moreover, this vitamin also has antioxidant properties (Joshi et al., 2001; Racek et al., 2005; Patro et al., 2006; Au-Yeung et al., 2006). In addition, it has been reported that folic acid has an important role on DNA stability maintenance, preventing uracil misincorporation into DNA, single- and double-strand DNA breaks and micronucleus formation (Fenech et al., 2005; Fenech, 2001).

In the present study we evaluated the effect of chronic hyperhomocysteinemia on some parameters of oxidative damage, namely total radical-trapping antioxidant potential (TRAP) and activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPx, EC 1.11.1.9), as well as on DNA damage (comet assay and micronucleus frequency) in parietal cortex, blood and/or plasma of rats. We also evaluated folic acid effects on biochemical alterations elicited by chronic hyperhomocysteinemia.

2. Materials and methods

2.1. Animals and reagents

Ninety-seven Wistar rats were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 80-23, revised 1996) was followed in all experiments.

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Folic acid and homocysteine chronic treatment

Folic acid and D,L-Hcy were dissolved in 0.85% NaCl solution and buffered to pH 7.4. Hcy solution was administered subcutaneously twice a day at 8 h intervals from their 6th to their 28th day-of-age. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Streck et al., 2002). During the first week of treatment, animals received 0.3 μmol Hcy/g body weight. In the second week, 0.4 μmol Hcy/g body weight was administered to the animals, and in the last week rats received 0.6 μmol Hcy/g body weight. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those found in homocystinuric patients. Folic acid solution (0.011 μmol folic acid/g of weight body) was injected intraperitoneally, once a day, from their 6th to their 28th day-of-age (Lalonde et al., 1993; Matté et al., 2007). Control animals received saline solution in the same volumes as those applied to Hcy- and folic acid-treated rats.

The rats were sacrificed by decapitation without anesthesia 12 h after the last injection. The brain was quickly removed and parietal cortex was dissected. Total blood was collected using heparin as anticoagulant, and processed as quickly as possible, to avoid the damage associated with storage.

2.3. Tissue preparation

For acquisition of the plasma, whole blood was centrifuged at 1000 × *g* and the plasma was immediately removed by aspiration to determination of TRAP.

Parietal cortex was homogenized 1:5 (w/v) in 0.1 M glycine buffer, pH 8.6 to TRAP assay, or 1:10 (w/v) in 20 mM sodium phosphate buffer containing 140 mM KCl, pH 7.4 to antioxidant enzymes determination. The homogenate was centrifuged at 800 × *g* for 10 min. Supernatant was taken to assays.

For comet assay, a coronal slice (0.4 mm) of parietal cortex was placed in 0.5 mL of cold phosphate-buffered saline and minced into fine pieces in order to obtain a cellular suspension.

2.4. Total radical-trapping antioxidant potential assay

TRAP represents the total non-enzymatic antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis (2-amidinopropane) (ABAP) at room temperature (Lissi et al., 1992). Four milliliters of 10 mM ABAP were added to the vial and the background chemiluminescence was measured. Ten microliters of 4 mM luminol were then added and the chemiluminescence was measured. This was considered to be the initial value. Ten microliters of 300 μM trolox or supernatant or plasma were added and chemiluminescence was measured until it reached the initial levels. The addition of trolox or sample to the incubation medium reduced the chemiluminescence. The time necessary to return to the levels presented before the addition was considered to be the induction time, which is directly proportional to the antioxidant capacity of the tissue and was compared to the induction time of trolox. The results are reported as nanomole of trolox per milligram of protein.

2.5. Antioxidant enzymes

2.5.1. Superoxide dismutase assay

For parietal cortex, SOD activity assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is 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 per milligram of protein.

In blood samples, SOD activity was determined spectrophotometrically based on its ability to inhibit the autoxidation of epinephrine to adrenochrome at alkaline pH, according to Misra and Fridovich (1972). SOD activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit.

2.5.2. Catalase assay

CAT activity determination, in blood or parietal cortex samples, 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, 100 mM potassium phosphate buffer with 1 mM EDTA, pH 7.0 (Aebi, 1984). One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute, and the specific activity is reported as *k* per gram of protein (blood) or units per milligram of protein (parietal cortex).

2.5.3. Glutathione peroxidase assay

GPx activity, in blood and parietal cortex samples, was measured using *tert*-butyl-hydroperoxide as substrate. NADPH disappearance was monitored spectrophotometrically at 340 nm. The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl-hydroperoxide and 0.1 mM NADPH (Paglia and Valentine, 1967; Wendel, 1981). One GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as nanomole NAD per minute per milliliter (blood) or units per milligram of protein (parietal cortex).

2.6. Comet assay

The comet assay was carried out as described by Tice et al. (2000). Under alkaline conditions the comet assay detects DNA single- and double-strand breaks and alkali-labile sites. Slides were prepared using 5 μL of total blood or a cellular suspension from parietal cortex embedded in 95 μL of 0.75% low melting point agarose (Gibco-BRL). The mixture was added to a fully frosted microscope slide coated with 300 μL of 1% normal melting agarose (Gibco-BRL). After solidification, the slides were placed in frosty lysis buffer [2.5 M NaCl, 100 mM ethylene diamine tetra-acetic acid (EDTA) and 10 mM Tris, pH 10.0–10.5 with 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO)] for a minimum of 1 h and a maximum of 7 days, at 4 °C. Subsequently, the slides were incubated in frosty alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 30 min. DNA electrophoresis was performed using 25 V (0.90 V/cm) and 300 mA for 30 min. The slides were then neutralized (0.4 M Tris, pH 7.5) and stained with silver nitrate. The samples were evaluated by blind analysis.

Negative and positive controls were used for each electrophoresis assay in order to ensure the reliability of the procedure. One hundred cells (50 from each of two replicate slides) were randomly selected from each animal. Cells were scored visually according to tail size into five classes ranging from undamaged (0) to maximally damaged (4), resulting in a single DNA damage score for each animal, and consequently each studied group. Therefore, the damage index can range from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damage, 100 cells × 0). The index of damage was calculated based on number of cells with tail versus those with no tails.

2.7. Micronucleus test

Micronucleus test was performed as described by Trippi et al. (2001). For each blood sample, duplicate cells cultures were set up in culture flasks by adding 0.3 mL of whole blood to 5 mL of RPMI 1640 medium containing 20% fetal calf serum and

1% (v/v) phytohemagglutinin. The flasks were incubated at 37 °C for 44 h before adding 5 μg/mL of cytochalasin B and continuing incubation until the total incubation time reached 72 h. After incubation, the cells were harvested by centrifugation at 800 × *g* for 8 min, recentrifuged, fixed in 3:1 (v/v) methanol/acetic acid, placed onto a clean microscope slide and stained with 5% (v/v) Giemsa. For each blood sample, 1000 binucleated cells (i.e. 500 from each of the two slides prepared from the duplicate cultures) were scored for micronuclei presence, using bright-field optical microscopy at a magnification of 200–1000×. All sides slides were coded blinded.

2.8. Protein determination

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

2.9. Statistical analysis

Data were analyzed by Student's *t* test, Kruskal–Wallis non-parametric test, or one-way analysis of variance (ANOVA) followed by Duncan's multiple range test when the *F* value was significant, as indicated on captions. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Folic acid prevents reduction in total radical-trapping antioxidant potential in parietal cortex and plasma elicited by chronic homocysteine administration in rats

Fig. 1 shows that Hcy administration reduced antioxidant potential, TRAP, in parietal cortex by 30% (A) [$F(3,16) = 5.00$; $p < 0.05$] and in plasma by 40% (B) [$F(3,14) = 5.87$; $p < 0.01$]. *Post hoc* analysis showed that folic acid treatment was ineffective *per se*, however prevented the reduction in TRAP elicited by Hcy chronic administration in rats.

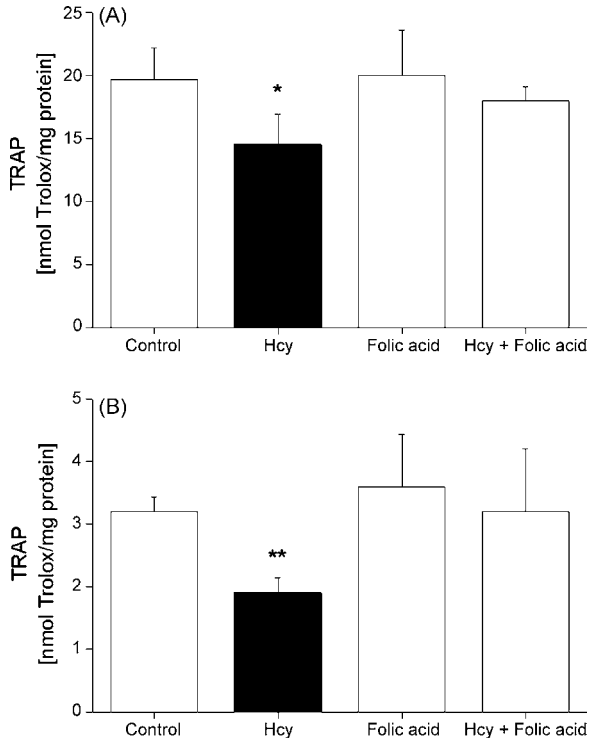

Fig. 1. Effect of homocysteine, folic acid, and/or homocysteine plus folic acid administration on total radical-trapping antioxidant potential in parietal cortex (A) and plasma (B) of rats. Results are expressed as mean ± S.D. for four to five animals in each group. Different from control, $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Duncan's multiple range test). Hcy: homocysteine.

3.2. Folic acid prevents alterations in antioxidant enzymes in parietal cortex and blood from rats subjected to chronic hyperhomocysteinemia in rats

The effect of chronic hyperhomocysteinemia on antioxidant enzymes (SOD, CAT and GPx) in parietal cortex and blood was also evaluated. Fig. 2 shows that Hcy significantly reduced the activities of CAT [$F(3,19) = 4.19$; $p < 0.05$] and GPx (C) [$F(3,16) = 4.83$; $p < 0.05$] around 25% in rat parietal cortex. SOD activity (A) was not altered by Hcy administration [$t(6) = -1.56$; $p > 0.05$]. *Post hoc* analysis showed that folic acid concurrent administration *per se* did not alter the activities of CAT and GPx, but prevented the inhibition of these enzymes caused by Hcy.

Fig. 3 shows that Hcy administration increased by 58% SOD activity (A) [$F(3,14) = 3.62$; $p < 0.05$], and by 268% CAT activity (B) [$F(3,12 = 11.2$; $p < 0.01$] in blood of rats. GPx (C) was not altered by chronic hyperhomocysteinemia [$t(10) = 0.00$; $p > 0.05$] in blood.

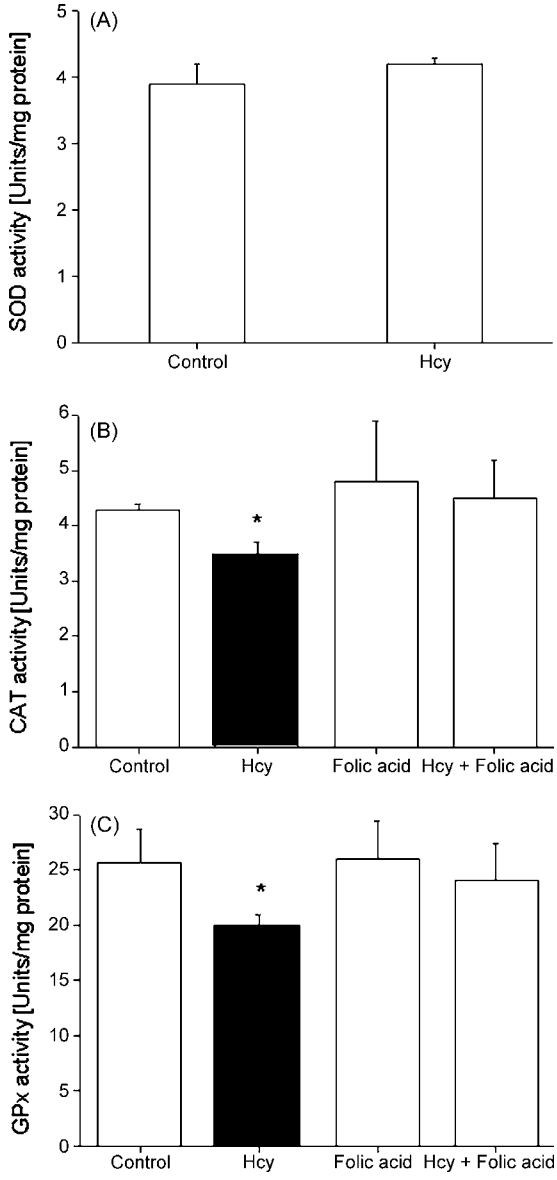

Fig. 2. Effect of homocysteine, folic acid, and/or homocysteine plus folic acid administration on superoxide dismutase (A), catalase (B), and glutathione peroxidase (C) in parietal cortex of rats. Results are expressed as mean ± S.D. for four to six animals in each group. Different from control, $^{*}p < 0.05$ (Student's *t*-test, or one-way ANOVA followed by Duncan's multiple range test). Hcy: homocysteine.

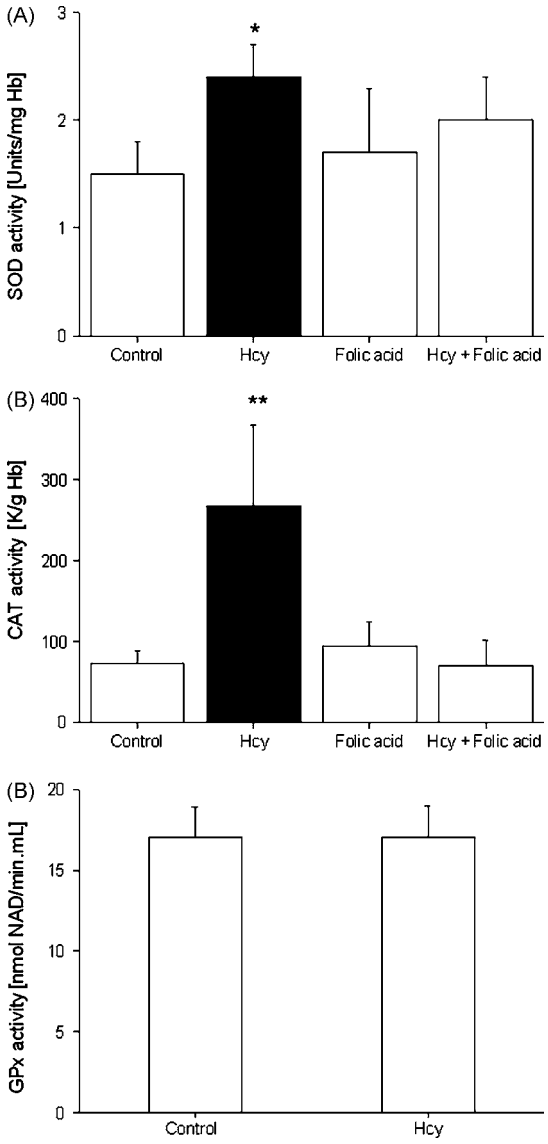

Fig. 3. Effect of homocysteine, folic acid, and/or homocysteine plus folic acid administration on superoxide dismutase (A), catalase (B), and glutathione peroxidase (C) in blood of rats. Results are expressed as mean ± S.D. for four to six animals in each group. Different from control, $^{**}p < 0.01$, $^{*}p < 0.05$ (one-way ANOVA followed by Duncan's multiple range test, or Student's *t*-test). Hcy: homocysteine.

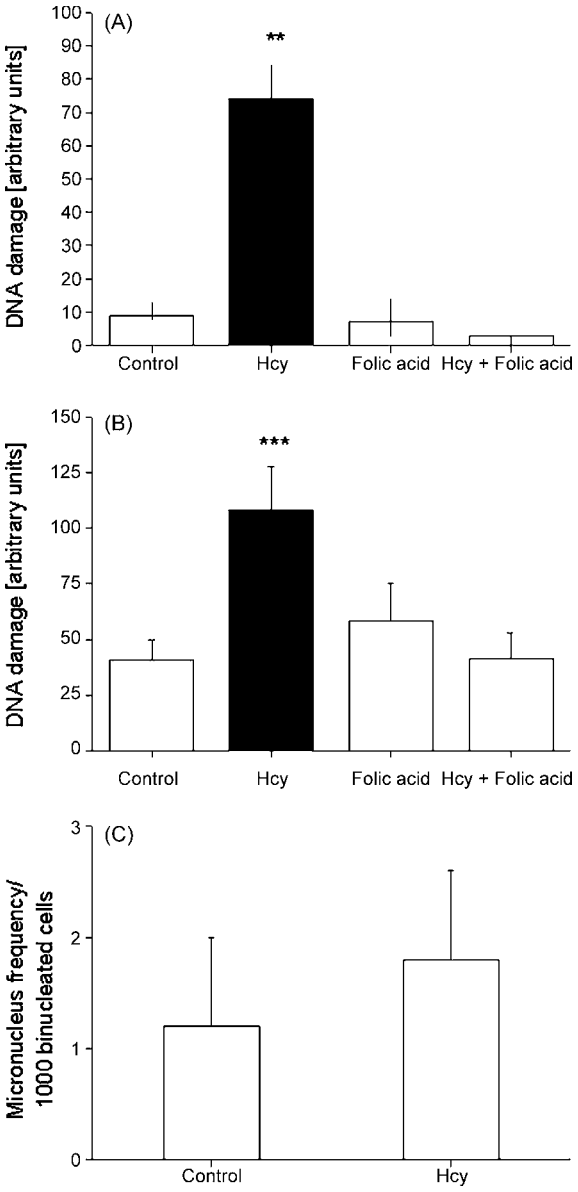

Fig. 4. Effect of homocysteine, folic acid, and/or homocysteine plus folic acid administration on DNA damage (comet assay) in parietal cortex (A) and blood (B), and on micronucleus frequency in blood (C) of rats. Results are expressed as median ± interquartile range or mean ± S.D. for five to seven animals in each group. Different from control, $^{**}p < 0.01$; $^{***}p < 0.001$ (Kruskal–Wallis test, one-way ANOVA followed by Duncan's multiple range test, or Student's *t*-test). Hcy: homocysteine.

Post hoc analysis showed that folic acid per se did not change SOD and CAT activities, but prevented the activation of these enzymes caused by Hcy.

3.3. Folic acid prevents DNA damage in parietal cortex and blood induced by chronic homocysteine administration in rats

We also evaluated the effect of chronic Hcy administration on an index of DNA damage, measured by comet assay, in parietal cortex and blood of rats. As can be seen in Fig. 4, Hcy increased significantly DNA damage by 1190% in parietal cortex [$H = 11.72$; $p < 0.01$] (Fig. 4A) and by 170% in blood [$F(3,20) = 27.81$; $p < 0.001$] (Fig. 4B). *Post hoc* analysis showed that folic acid administration did not affect this parameter, but prevented DNA damage caused by hyperhomocysteinemia in both tissues.

In order to evaluate whether DNA damage was permanent after cell division, we also investigated the effect of Hcy on the micronucleus test. Fig. 4C shows that hyperhomocysteinemia did not alter micronucleus frequency in blood of rats [$t(8) = -1.13$; $p > 0.05$].

4. Discussion

Hyperhomocysteinemia is a consequence of a wide range of determinants: genetic, such as homocystinuria by CBS deficiency; physiological, such as diet rich in methionine and/or poor in B-vitamins; pathological, such as renal disease; or medications, such as antifolate drugs (Perry, 1999; Eikelboom et al., 1999). Moreover, increased levels of Hcy also appear to be associated with neurodegenerative and vascular disorders, and some types of cancer (Welch and Loscalzo, 1998; Eikelboom et al., 1999; Diaz-Arrastia, 2000; Mattson et al., 2002; Mattson and Haberman, 2003;

Slupphaug et al., 2003; Sachdev, 2004; Ullegaddi et al., 2006). The underlying pathophysiological mechanisms of hyperhomocysteinemia in these disorders are still not known.

We have developed a chemical experimental rat model of hyperhomocysteinemia (Streck et al., 2002), where plasma Hcy concentration achieve levels similar to those found in human homocystinuria (Mudd et al., 2001). In order to mimetic homocystinuria, we started homocysteine chronic administration in postnatal day 6, because rat neurodevelopment is equivalent to that of the human brain at birth (Clancy et al., 2007). Using this model, we have verified the effect of Hcy on biochemicals and behavioural parameters in rats. Besides, we also have evaluated the effect of concurrent administration of folic acid on memory deficits observed in rats subjected to chronic hyperhomocysteinemia (Matté et al., 2007).

Since previous studies showed that acute hyperhomocysteinemia reduced TRAP and CAT activity in hippocampus, which were prevented by antioxidants (Wyse et al., 2002), in the present study we extend our investigation, evaluating the effect of chronic hyperhomocysteinemia on antioxidant defenses. First, we investigated the effect of chronic Hcy administration on TRAP, an index of antioxidant capacity, in parietal cortex and plasma of rats. Results showed that Hcy reduced non-enzymatic antioxidant potential in tissues, and that folic acid administration prevented such effects.

Next, we also evaluated the effect of chronic hyperhomocysteinemia on the activities of antioxidant enzymes, which compose an efficient system responsible for removing ROS (Halliwell, 2001; Halliwell and Gutteridge, 2007). Results showed that Hcy administration inhibited CAT and GPx in parietal cortex of rats. We believe that this imbalance between antioxidant enzymes probably alter ROS elimination, promoting H_2O_2 rise in parietal cortex. By the Fenton reaction, this reactive specie could generate $OH^{\bullet}$, which is the harmful oxygen radical that might injury DNA, proteins and lipids (Halliwell, 2001; Halliwell and Gutteridge, 2007). We also observed that folic acid prevented the reduction of antioxidant enzymes in parietal cortex of hyperhomocysteinemic rats. In blood we observed a different result, since Hcy increased SOD and CAT activities. It is well known that SOD and CAT own sequential functions in ROS removing, by $O_2^{\bullet -}$ dismutation, followed by H_2O_2 conversion to H_2O and O_2, respectively (Halliwell and Gutteridge, 2007), thus we believe that CAT activity was increased by a compensatory effect, to remove the H_2O_2 in excess generated by SOD increases in blood. On the other hand, it has been reported that Hcy can regulate protein turnover and gene expression, including antioxidant enzymes (Stern et al., 2004; Sharma et al., 2006). These effects of Hcy could be responsible, at least in part, by alterations in the activities of antioxidant enzymes observed in our study. However, we cannot discard that the increase in SOD and CAT activities was a consequence of ROS generated by Hcy autoxidation (Ho et al., 2002; Dayal et al., 2004; Faraci and Lentz, 2004). This hypothesis could be associated with folic acid results since this vitamin is considered antioxidant and in our present study we observed that it prevented the increases of CAT and SOD activities.

Since ROS are the main responsible for DNA injury (Halliwell, 2001; Slupphaug et al., 2003; Higuchi, 2003; Warner et al., 2004; Cherubini et al., 2005), we also investigated the effect of chronic hyperhomocysteinemia on DNA damage assays. Our results showed that chronic hyperhomocysteinemia induced DNA damage in parietal cortex and blood, evaluated by comet assay, which, under alkaline conditions, detects DNA single- and double-strand breaks, and alkali-labile sites (Tice et al., 2000). Folic acid administration prevented such effect. Besides, we evaluated some parameters of protein damage, namely total thyol content in parietal cortex and carbonyl content in blood of rats subjected to Hcy chronic administration. Hcy did not alter these parameters (data not shown).

Previous studies show that Hcy *in vitro* induced oxidative stress in parietal cortex (Matté et al., 2004) and hippocampus (Streck et al., 2003), increasing lipid peroxidation and reducing TRAP. Recently, Matté et al. (2007) showed that Hcy chronic administration increased lipid peroxidation in parietal cortex, and concurrent administration of folic acid prevented such effect. Taken our results together, we could suggest that Hcy induces oxidative stress in brain tissue.

Our results agree with those of Kruman et al. (2000), who reported that Hcy induced apoptosis in rat hippocampal neurons, as a consequence of DNA strand breaks and oxidative stress. Picerno et al. (2007) showed that Hcy-treated human peripheral blood lymphocytes in culture presented increase in DNA damage and in micronucleus frequency, altering immune function. However, we did not discard other ways of action, considering that DNA strand breakage could be caused by nucleases activated by Ca^{2+} and/or by ROS, mainly $OH^{\bullet}$ formed by reaction of H_2O_2 with DNA-bound metal ions (Darley-Usmar and Halliwell, 1996). Otherwise, Hcy could generate ROS by autoxidation, producing $O_2^{\bullet -}$ and H_2O_2 (Ho et al., 2002; Dayal et al., 2004; Faraci and Lentz, 2004). This amino acid also is implicated in activation of NMDA and group I metabotropic glutamatergic receptors present in neurons (Jara-Prado et al., 2003; Boldyrev et al., 2005; Zieminska and Lazarewicz, 2006), resulting in oxidative stress, endonucleases activation, modulation and gene expression disturbances (Higuchi, 2003; Boldyrev et al., 2005). In addition, there is evidence that neurotoxicity elicited by Hcy included glutamatergic receptors activation, following by enhanced Ca^{2+} influx, DNA damage, activation of apoptotic signaling cascades, and endoplasmic reticulum stress (Mattson and Shea, 2003).

We also demonstrated that folic acid prevents oxidative stress induced by Hcy administration. This B-vitamin has intrinsic antioxidant properties, acting as a Fenton-modulator (Patro et al., 2006), and/or scavenging ROS, such as $OH^{\bullet}$ (Joshi et al., 2001). Au-Yeung et al. (2006) showed that folic acid prevented the generation of $O_2^{\bullet -}$ caused by Hcy in macrophages. On the other hand, folic acid has an important role in DNA synthesis and stability, preventing uracil misincorporation, DNA hypomethylation and DNA strand breaks (Fenech, 2001; Fenech et al., 2005). Although in our study we did not observe any effect of folic acid *per se* on antioxidant defenses (TRAP and antioxidant enzymes), many authors report that this vitamin increases enzymatic and non-enzymatic antioxidant defenses in hyperhomocysteinemic patients (Mayer et al., 2002; Abdelfatah et al., 2002; Racek et al., 2005). Previous studies showed that folic acid administration did not reduce Hcy levels in brain and plasma from hyperhomocysteinemic rats, suggesting that this vitamin action in independent of Hcy-lowering effect (Matté et al., 2007). These results agree with those of other investigators (Achón et al., 2000; Bernasconi et al., 2006; Carnicer et al., 2007).

In conclusion, we showed that Hcy chronic administration provoked DNA damage and altered antioxidant defenses (enzymatic and non-enzymatic), characterizing this amino acid as an oxidative stressor. Concurrent folic acid administration prevented Hcy effects, possibly by its antioxidant features, or by DNA stability maintenance. Considering that oxidative stress elicited by Hcy could contribute to pathophysiology of disorders that accumulate this amino acid such as metabolic, neurodegenerative, neuropsychiatric, and vascular disorders, and if confirmed in human beings, we might propose the use of folic acid as an adjuvant therapy in hyperhomocysteinemic patients.

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Capítulo II

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

[b] *Departamento de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil*

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[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil
[b] Departamento de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil

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ABSTRACT

Hyperhomocysteinemia has been related to various diseases, including homocystinuria, neurodegenerative and hepatic diseases. In the present study we initially investigated the effect of chronic homocysteine administration on some parameters of oxidative stress, named total radical-trapping antioxidant potential, total antioxidant reactivity, catalase activity, chemiluminescence, thiobarbituric acid-reactive substances, and total thiol content in liver of rats. We also performed histological analysis, evaluating steatosis, inflammatory infiltration, fibrosis, and glycogen/glycoprotein content in liver tissue sections from hyperhomocysteinemic rats. Finally, we evaluated the activities of aminotransferases in liver and plasma of hyperhomocysteinemic rats. Wistar rats received daily subcutaneous injection of Hcy from their 6th to their 28th day of life. Twelve hours after the last injection the rats were sacrificed, liver and plasma were collected. Hyperhomocysteinemia decreased antioxidant defenses and total thiol content, and increased lipid peroxidation in liver of rats, characterizing a reliable oxidative stress. Histological analysis indicated the presence of inflammatory infiltrate, fibrosis and reduced content of glycogen/glycoprotein in liver tissue sections from hyperhomocysteinemic rats. Aminotransferases activities were not altered by homocysteine. Our data showed a consistent profile of liver injury elicited by homocysteine, which could contribute to explain, at least in part, the mechanisms involved in human liver diseases associated to hyperhomocysteinemia.

1. Introduction

Tissue accumulation of homocysteine (Hcy) occurs in homocystinuria, a metabolic disease characterized biochemically by cystathionine β-synthase (CBS, EC 4.2.1.22) deficiency and clinically by mental retardation, vascular problems, skeletal abnormalities, and hepatic compromise, with fatty accumulation and cirrhosis (Mudd et al., 2001; Snyderman, 2006). Hyperhomocysteinemia is also implicated in hepatic disorders, such as alcoholic liver disease (Roblin et al., 2007), cirrhosis (Bosy-Westphal et al., 2001), steatosis, and fibrosis (Adinolfi et al., 2005; Ventura et al., 2005). This correlation is pertinent, as far as the liver is central in Hcy metabolism (Finkelstein, 1998; Brosnan et al., 2004).

Chronic hepatic insult leads the recruitment of inflammatory cells, cytokines production and reactive oxygen species (ROS) generation, which appear to have a central role in development of steatosis and fibrosis (Lieber, 1997; Kershenobich Stalnikowitz and Weissbrod, 2003; Urtasun and Nieto, 2007; Halliwell and Gutteridge, 2007; Mantena et al., 2008). It has been described that oxidative stress can cause fibrosis (Parola et al., 1993; Kawada, 1997; Prosser et al., 2006; Kisseleva and Brenner, 2007; Urtasun and Nieto, 2007). Studies also show that activated hepatic stellate cells (HSC) secretes inflammatory cytokines and up-regulates adhesion molecules, that promote immune cell attraction, producing a loop of autocrine and paracrine activation (Kisseleva and Brenner, 2007; Friedman, 2008). Poli and Parola (1997) established the association between lipid peroxidation and fibrogenesis, where products of lipid peroxidation appear to modulate the activity of phagocytes and mainly extracellular matrix-producing cells, increasing collagen synthesis. In this regard, it has been reported

* Corresponding author. Tel.: +55 51 3308 5573; fax: +55 51 3308 5535.
E-mail address: wyse@ufrgs.br (Angela T.S. Wyse).

Abbreviations: ABAP, 2,2′-azo-bis-(2-amidinopropane); ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; CBS, cystathionine β-synthase; CPS, counts per second; DTNB, 5′-dithiobis-(2-nitrobenzoic acid); Hcy, homocysteine; HSC, hepatic stellate cells; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinases; NFκB, nuclear factor kappa B; PAS, periodic acid-Schiff; ROS, reactive oxygen species; SPSS, statistical package for the social sciences; TAR, total antioxidant reactivity; TBARS, thiobarbituric acid-reactive substances; TIMPs, inhibitors of MMPs; TRAP, total radical-trapping antioxidant potential.

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that Hcy elicits hepatic damage in experimental models by three mechanisms, named oxidative stress, endoplasmic reticulum stress, and activation of proinflammatory factors (Ji and Kaplowitz, 2004; Robert et al., 2005).

Liver injury could be identified by serum markers, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are increased as a result of hepatic necrosis (Ozer et al., 2008). Hcy has been related to hepatotoxic conditions in numerous reports, which have showed a positive correlation between hyperhomocysteinemia and plasma aminotransferases activities in clinical and experimental studies (Huang et al., 2001; Woo et al., 2006; Frelut et al., 2006). In this context, an increase of ALT and AST activities was observed in serum of hyperhomocisteinemic rats (Woo et al., 2006) and of ALT in plasma hyperhomocysteinemic patients (Frelut et al., 2006).

In the present study we investigated the effect of chronic hyperhomocysteinemia on some parameters of oxidative stress, named total radical-trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), catalase activity (CAT, EC 1.11.1.6), induced chemiluminescence, thiobarbituric acid-reactive substances (TBARS), and total thiol content in liver of rats. We also performed histological analysis, evaluating steatosis, inflammatory infiltration, fibrosis, and glycogen/glycoprotein content in liver tissue sections of hyperhomocysteinemic rats. Finally, we assessed the activities of aminotransferases (ALT and AST) in liver and plasma of Hcy-treated rats.

2. Materials and methods

2.1. Animals and reagents

Ninety-six male or female Wistar rats (6th day-of-age) were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 80-23, revised 1996) was followed in all experiments.

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA; except 2,2′-azo-bis-(2-amidinopropane) (ABAP), which was purchased from Wako Chemicals (USA).

2.2. Chemically induced chronic hyperhomocysteinemia

D,L-Hcy was dissolved in 0.85% NaCl solution and buffered to pH 7.4. Hcy solution was administered subcutaneously twice a day at minimum 8 h interval from their 6th to their 28th day-of-age (51 rats). Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Streck et al., 2002). During the first week of treatment, animals received 0.3 μmol Hcy/g body weight. In the second week, 0.4 μmol Hcy/g body weight was administered to the animals, and in the last week rats received 0.6 μmol Hcy/g body weight. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those found in homocystinuric patients (Mudd et al., 2001). Control animals (45 rats) received saline solution in the same volumes as those applied to Hcy-treated rats.

2.3. Tissue preparation

2.3.1. Biochemical parameters

The rats were sacrificed by decapitation without anesthesia 12 h after the last injection. Blood was collected using heparin as anticoagulant, and the plasma was separated by centrifugation, at 800 × *g* for 5 min at 25 °C, for aminotransferases activities determination. The liver was quickly removed and processed as follows.

To oxidative stress parameters determination, the liver was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 800 × *g* for 10 min at 4 °C, to discard nuclei and cell debris. The pellet was discarded and the supernatant was taken to biochemical assays. The homogenates used were from individual animals, and they were never pooled. All experiments were repeated with different animals.

To aminotransferases activities measure, the liver was homogenized in 20 volumes (1:20, w/v) of 0.32 M sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4. The homogenate was centrifuged at 800 × *g* for 10 min at 4 °C, the pellet was discarded and the supernatant was centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant of the second centrifugation, containing cytosol and other cellular components as endoplasmic reticulum, was collected as cytosolic fraction. The pellet, containing mitochondria, myelin, synaptosomes, and membrane fragments, was washed twice, and ressuspended in 0.32 M sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4. The mitochondrial fraction was frozen and thawed three times for the enzymatic assay. Cytosolic and mitochondrial fractions were stored for no longer than one week at −70 °C when the assay was not carried out immediately.

2.3.2. Histological analysis

For histological analysis, 12 h after the last Hcy injection, a group of rats were anesthetized with a mixture of ketamine and xylazine (75 and 10 mg/kg body weight, respectively), and submitted to transcardiac perfusion with fresh 0.85% saline followed by fresh 4% formaldehyde solution, pH 7.4. The liver was removed and post-fixed in 4% formaldehyde solution.

2.4. Total radical-trapping antioxidant potential assay

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by ABAP thermolysis (Lissi et al., 1992; Evelson et al., 2001) in a Wallac 1409 Scintillation Counter. Three mL of ABAP 10 mM, dissolved in 50 mM sodium phosphate buffer pH 7.4, plus 10 μL of luminol (5.6 mM) were added to a glass scintillation vial, and the initial chemiluminescence was measured. Ten microlitres of 160 μM Trolox (water-soluble α-tocopherol analogue, used as standard) or 10 μL of tissue supernatant were then added to that vial, producing a decrease in the initial chemiluminescence value. This value is kept low until the antioxidants present in the sample are depleted, then chemiluminescence 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, so TRAP represents the amount (quantity) of non-enzymatic antioxidants present in the sample. The results were calculated as nmol Trolox/mg protein.

2.5. Total antioxidant reactivity assay

TAR was determined by measuring the luminol chemiluminescence intensity induced by ABAP thermolysis (Lissi et al., 1995) using a Wallac 1409 Scintillation Counter. The background chemiluminescence was measured by adding 4 mL of 2 mM ABAP, prepared in 0.1 mM glycine buffer, pH 8.6, plus 15 μL of luminol (4 mM) into a glass scintillation vial. Ten microlitres of 20 μM Trolox or tissue supernatant was then added and the chemiluminescence was measured during 60 s to evaluate how fast it falls. This velocity of reduction in luminol intensity reflects the tissue capacity to promptly react against an enhanced free radical production. TAR represents not the amount but the reactivity (quality) of non-enzymatic antioxidants present in the sample. The results were calculated as nmol Trolox/mg protein.

2.6. Catalase assay

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 μmol of H_2O_2 consumed per minute and the specific activity is represented as CAT units/mg protein.

2.7. Induced chemiluminescence assay

Samples were assayed for chemiluminescence in a dark room using a beta liquid scintillation spectrometer Tri-Carb 2100TR (González-Flecha et al., 1991). The background chemiluminescence was measured for 5 min in vials containing 1.8 mL of the same buffer used for homogenization (20 mM sodium phosphate buffer pH 7.4 with 140 mM KCl) and 100 μL of 3 mM tert-butyl hydroperoxide. An aliquot of 100 μL of liver supernatant was added, the mixture was incubated at 30 °C for 30 min in the dark, and chemiluminescence was measured for 10 min at room temperature. The background chemiluminescence was subtracted from the total value. Chemiluminescence was calculated as counts per second (CPS)/mg protein.

2.8. Thiobarbituric acid-reactive substances assay

TBARS were measured according to Ohkawa et al. (1979). Briefly, to glass tubes were added, in order of appearance: 500 μL of homogenate; 50 μL of SDS 8.1%; 1500 μL of 20% acetic acid in aqueous solution (v/v) pH 3.5; 1500 μL of 0.8% thiobarbituric acid; and 700 μL of water. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 h. The mixture was allowed to cool on water for 5 min, and was centrifuged at 750 × *g* for 10 min at 25 °C. The resulting pink stained TBARS obtained were determined spectrophotometrically at 535 nm in a Beckman DU®640 Spectrophotometer. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard, being subjected to the same treatment as that of the samples. TBARS were calculated as nmol TBARS/mg protein.

2.9. Total thiol content assay

This assay is based on the reduction of 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) by thiols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov

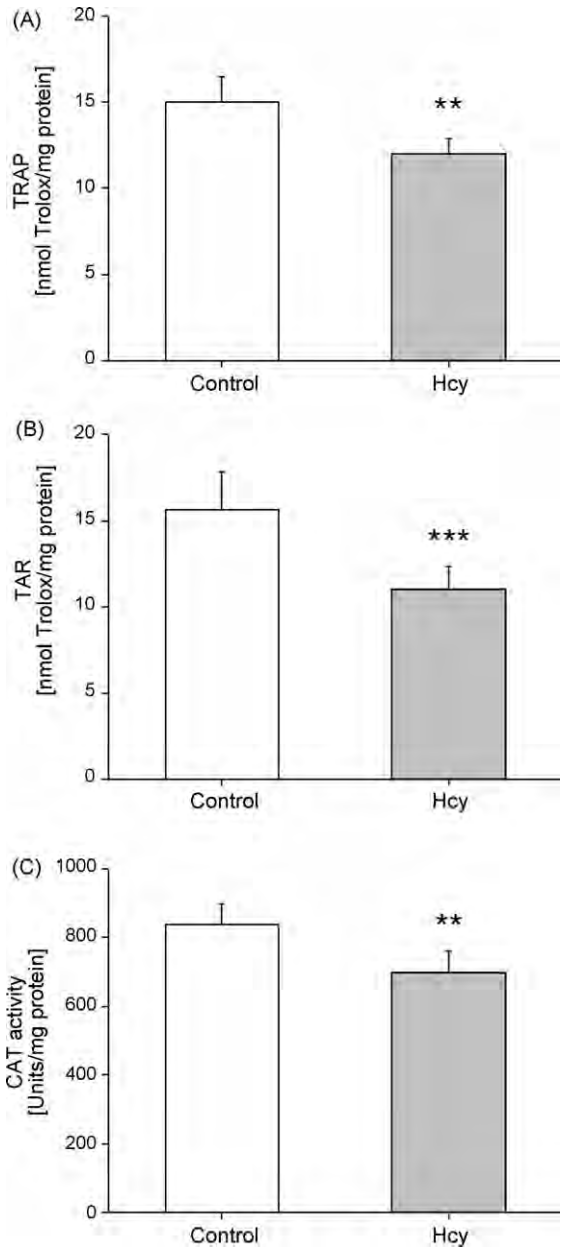

Fig. 1. Effect of homocysteine chronic administration on total radical-trapping antioxidant potential (A), total antioxidant reactivity (B), and catalase activity (C) in liver of rats. Results are expressed as mean ± S.D. for 7–8 animals in each group. Different from control, $^{**}p < 0.01$; $^{***}p < 0.001$ (Student's t test). Hcy: homocysteine.

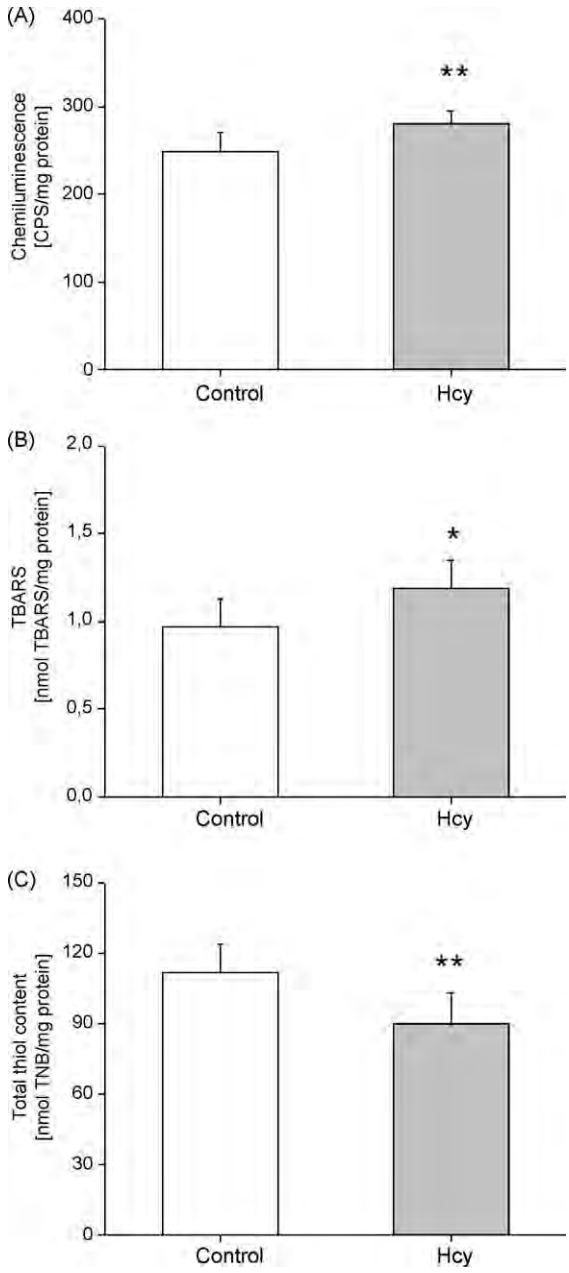

Fig. 2. Effect of homocysteine chronic administration on induced chemiluminescence (A), thiobarbituric acid-reactive substances (B), and total thiol content (C) in liver of rats. Results are expressed as mean ± S.D. for 7–8 animals in each group. Different from control, $^{*}p < 0.05$; $^{**}p < 0.01$ (Student's t test). Hcy: homocysteine.

and Markesbery, 2001). Briefly, 50 μL of homogenate were added to 1 mL of phosphate saline buffer pH 7.4 containing 1 mM EDTA. Then 30 μL of 10 mM DTNB, prepared in a 0,2 M potassium phosphate solution pH 8.0, were added. Subsequently, 30 min incubation at room temperature in a dark room was performed. Absorption was measured at 412 nm using a Beckman DU® 640 spectrophotometer. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

2.10. Histological analysis

Fragments from the central portion, left and right lobes of liver were processed and embedded in Paraplast®. Semi-serial 5 μm-thick sections were placed in glass slides and stained with hematoxylin/eosin for morphological analysis (Bancroft and Stevens, 1990). Picrosirius and periodic acid-Schiff (PAS) staining was performed for the analysis of fibrillar collagen and glycoproteins/glycogen content, respectively (Junqueira et al., 1979; Bancroft and Stevens, 1990). Pictures were taken using a CCD camera coupled to a Zeiss 135 inverted microscope. The software Adobe Photoshop was used to construct the panels.

2.11. Aminotransferases activities assays

ALT and AST activities were determined in plasma, and liver tissue (cytosolic and mitochondrial fractions) using a colorimetric test, from Labtest®. The α-cetoacid (pyruvate or oxaloacetate) produced by aminotransferase activity reacts with 2,4 dinitrophenyl hydrazine to give the corresponding hydrazone in alkaline medium. The resulting brown color was measured spectrophotometrically. Aminotransferases activities were expressed as Units/mg protein.

2.12. Protein determination

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

2.13. Statistical analysis

Data were analyzed by Student's t test. All analyses were performed using the statistical package for the social sciences (SPSS) software. Differences were considered statistically significant if $p < 0.05$.

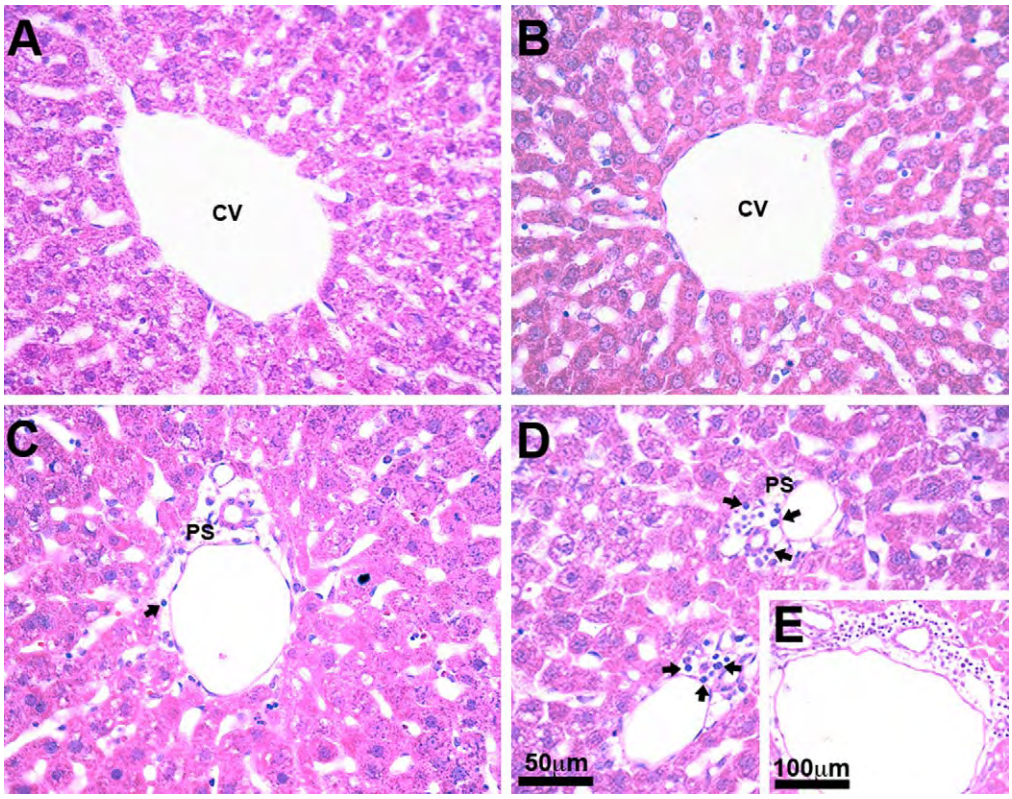

Fig. 3. Effect of homocysteine chronic administration on liver morphology assessed by hematoxylin/eosin staining. Representative photomicrographs from liver sections of control (A and C) and homocysteine-treated rats (B and D). In A and B, central region of hepatic lobules showing the central vein (cv), to where many sinusoids converge. In C and D, the portal space (ps) with its vascular components and bile duct. Homocysteine-treated animals exhibited high number of inflammatory cells (arrows) in the connective tissue present in portal space and around large vessels. Steatosis was not observed. The results are representative photomicrographs from three independent experiments. Scale bar = 50 μm.

3. Results

3.1. Homocysteine chronic administration reduced antioxidant defenses and total thiol content, and increased lipid peroxidation in liver of rats

We investigated the effect of chronic hyperhomocysteinemia on non-enzymatic and enzymatic antioxidant defenses in liver. Fig. 1 shows that Hcy-treated rats present a reduction on total antioxidant potential, TRAP, in 20% (A; $t(13) = 4.21$; $p < 0.01$), and antioxidant reactivity, TAR, in 30% (B; $t(13) = 4.70$; $p < 0.001$) in liver. Hyperhomocysteinemia also reduced enzymatic antioxidant status, inhibiting hepatic CAT activity by 17% (C; $t(13) = 4.44$; $p < 0.01$).

Next, we evaluated the effect of Hcy chronic administration on lipid peroxidation and total thiol content in hepatic tissue. Fig. 2 shows that hyperhomocysteinemia increased by 13% chemiluminescence (A; $t(13) = -3.29$; $p < 0.01$), and by 23% TBARS (B; $t(13) = -2.59$; $p < 0.05$), indicating a consistent lipid peroxidation Hcy-induced effect in liver of rats. Hcy also modified the total thiol content of homogenates from liver, reducing in 20% sulphydryl content (C; $t(13) = 3.23$; $p < 0.01$), which could be related to protein oxidation and/or reduced thiol antioxidant status in cells.

3.2. Histological analysis revealed inflammatory infiltration, fibrosis and reduced glycoprotein/glycogen content in liver of hyperhomocysteinemic rats

In order to evaluate whether oxidative stress elicited by Hcy administration could result in hepatic morphological alterations, we performed histological analysis in liver slices stained by hematoxylin/eosin, Picrosirius red and PAS. Fig. 3 shows that hyperhomocysteinemic rats (B and D) exhibited increased number of inflammatory cells in the connective tissue present in the portal space and around large vessels, when compared to control rats (A and C). Steatosis was not observed in control or treated animals. In Fig. 4, we could observe that hyperhomocysteinemic rats (B and D) presented a significant increase in collagen fibers surrounding hepatocytes, and in the connective tissue present in portal space and around large vessels (arrows), when compared to control slices (A and C). These data suggest that Hcy administration promoted a strong alteration in collagen fibers content in liver, resulting in fibrosis. Fig. 5 shows that Hcy chronic administration (B and D) clearly reduced hepatic glycoproteins/glycogen content, stained by PAS, as compared to control animals (A and C).

3.3. Homocysteine chronic administration did not alter aminotransferases activities in liver neither in plasma of rats

Chronic hyperhomocysteinemia did not alter ALT activity in liver (cytosolic fraction: control = 134 ± 26.7, Hcy = 111 ± 53.9, $t(12) = 1.03$; $p > 0.05$; mitochondrial fraction: control = 8.34 ± 1.34, Hcy = 8.11 ± 1.05, $t(12) = 0.355$; $p > 0.05$) neither in plasma (control = 0.38 ± 0.07, Hcy = 0.37 ± 0.13, $t(12) = 0.177$; $p > 0.05$). Similarly, AST activity also was not altered in liver (cytosolic fraction: control = 305 ± 25.4, Hcy = 336 ± 36.8, $t(12) = -1.82$; $p > 0.05$; mitochondrial fraction: control = 57.3 ± 5.94, Hcy = 56.4 ± 6.00, $t(12) = 0.269$; $p > 0.05$) neither in plasma (control = 2.05 ± 0.55, Hcy = 2.00 ± 0.60, $t(12) = 0.152$; $p > 0.05$) of rats treated chronically with Hcy.

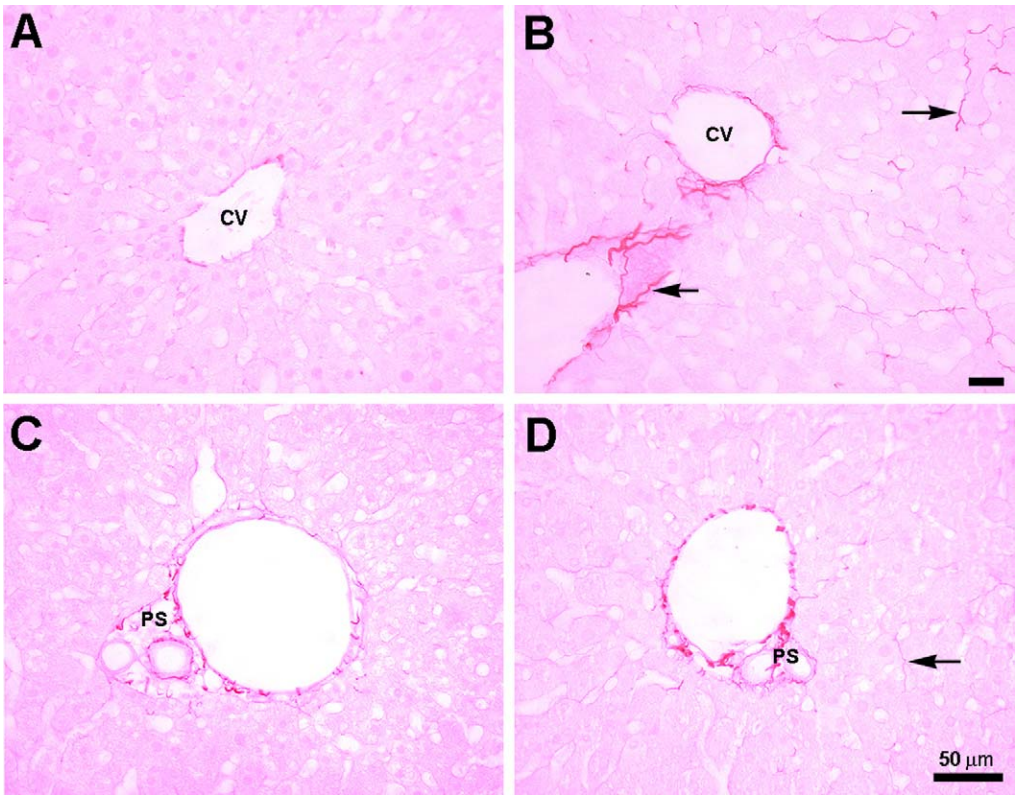

Fig. 4. Hepatic fibrillar collagen distribution after homocysteine chronic administration. Representative photomicrographs of liver sections processed for Picrosirius staining: control (A and C) and homocysteine-treated animals (B and D). In A and B, the central region of hepatic lobules showing the central vein (cv) with collagen fibers mostly in the subendothelial layer. In C and D, the portal space (ps). A thin net of collagen fibers (arrows) can be observed surrounding hepatocytes, while the connective tissue in the portal space presents high amount of fibrillar collagen. Homocysteine-treated animals (B and D) exhibited a significant increase in collagen fibers surrounding hepatocytes, as well as in the connective tissue present in portal space and around large vessels (arrows). The results are representative photomicrographs from three independent experiments. Scale bar = 50 μm.

4. Discussion

Hyperhomocysteinemia is a classical attribute of homocystinuria, nevertheless has been considered as a growing risk factor for neurodegenerative (Mattson and Haberman, 2003; Sachdev, 2004), cardiovascular (Clarke et al., 1999) and hepatic diseases (Bosy-Westphal et al., 2001; Adinolfi et al., 2005; Ventura et al., 2005; Roblin et al., 2007). Animal experimental models are significant approaches to study the pathological mechanisms related to these disorders. In this regard, our group has developed a chemical hyperhomocysteinemia in rats through Hcy administration from their 6th to their 28th day of life (Streck et al., 2002). Plasmatic total Hcy levels achieved are similar to those found in homocystinuric patients (Mudd et al., 2001). We have shown that rats subjected to this model present memory deficits in Morris water maze task, associated to inhibition of Na^+,K^+-ATPase activity (Matté et al., 2007), impaired energy metabolism and a substantial oxidative stress in parietal cortex (Matté et al., 2009).

In the present study we initially investigated the effect of Hcy on hepatic antioxidant status, TRAP and TAR. Both techniques evaluate the non-enzymatic antioxidant capacity, however while TRAP assess the total potential of the main antioxidants found in liver tissue (for example glutathione, uric acid, ascorbic acid, and α-tocopherol), TAR is a more useful index of antioxidant status because it considers not only the quantity, but also the reactivity of antioxidants (Lissi et al., 1995; Evelson et al., 2001). Our results showed for the first time that Hcy chronic administration decreased TRAP and TAR, suggesting that this amino acid causes a reduction on quantity and quality of non-enzymatic antioxidants in liver. We also verified the effect of Hcy on CAT, since this enzyme has the higher activity among the hepatic antioxidant enzymes (Polavarapu et al., 1998; Kasdallah-Grissa et al., 2007). We observed a significant inhibition of hepatic CAT activity in hyperhomocysteinemic rats. In agreement with our data, other studies suggest a negative correlation between plasma Hcy levels and CAT activity in liver of rats, pointing a significant reduction of hepatic antioxidant defenses (Woo et al., 2006; Chanson et al., 2007).

We also investigated the effect of hyperhomocysteinemia on some parameters of lipid damage and thiol status. Lipid peroxidation was assessed by chemiluminescence, which is an indicative of ROS generation throughout the process of peroxidation (González-Flecha et al., 1991), and by TBARS, which identify malondialdehyde, a final product of peroxidation (Ohkawa et al., 1979). Results showed that Hcy increased lipid peroxidation, identified by both assays used. In agreement, data from literature showed that hyperhomocysteinemia increased superoxide anion production, by NAD(P)H oxidase activation, and peroxynitrite formation, resulting in lipid peroxidation in liver of rats, which could explain many processes associated with Hcy-induced cell injury including apoptosis and inflammation in liver diseases (Ji and Kaplowitz, 2004; Woo et al., 2006). Hepatic lipid peroxidation appears to be strongly associated to Hcy levels. In this context, using a model of hyperhomocysteinemia induced by folic acid deprivation in rats, Huang et al. (2001) showed that the lipid peroxidation in liver of these animals is related to Hcy levels.

We also verified the effect of hyperhomocysteinemia on total thiol content, which was employed to verify protein damage to sulphydryl groups, as well as intracellular non-protein thiol levels, such as the antioxidant glutathione. We observed a consistent

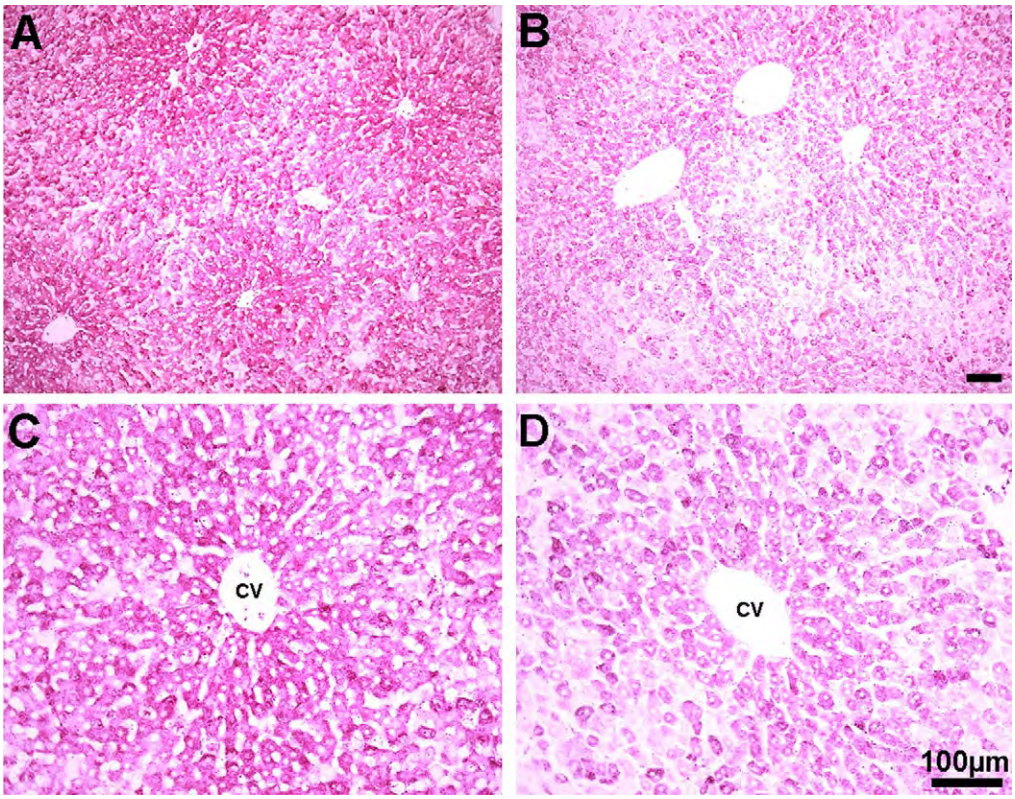

Fig. 5. Effect of homocysteine chronic administration on hepatic glycoproteins/glycogen assessed by periodic acid-Schiff staining. Representative photomicrographs from liver of control (A and C) and homocysteine-treated rats (B and D). In A and B, regular aspect of the liver with several hepatic lobules. In C and D, one hepatic lobule showing the central vein (cv); pericentral hepatocytes are located close to the cv, while periportal hepatocytes are more peripheral. PAS staining can be seen as purpur granules in the cytoplasm of hepatocytes, mostly corresponding to glycogen inclusions, and is more accentuated in pericentral hepatocytes when compared to periportal hepatocytes. In homocysteine-treated animals, although regional differences of glycogen distribution are still observed, the amount of glycogen is clearly lower. The results are representative photomicrographs from three independent experiments. Scale bar = 100 μm.

reduction in thiol content in liver tissue that supports the reduction in TRAP and TAR found in this work. Moreover, this data also could be a result of cross linking between products of lipid peroxidation and –SH groups (Comporti, 1993), which was also observed in liver of rats (Houglum et al., 1990). In addition, oxidation of thiol groups might be elicited by ROS, such as peroxynitrite (Radi et al., 1991), which are produced by Hcy autooxidation (Dayal et al., 2004; Faraci and Lentz, 2004) as well as by NAD(P)H oxidase activation in liver of hyperhomocysteinemic rats (Woo et al., 2006).

We next performed histological analysis in livers from hyperhomocysteinemic rats chronically-treated. Hematoxylin/eosin staining from Hcy-treated rats showed an increased number of inflammatory cells in the connective tissue within the portal space and surrounding large vessels, when compared to control rats. Recently, Woo et al. (2008) showed that hyperhomocysteinemia, induced by high-methionine diet for four weeks, elevated the expression and the protein synthesis of monocyte chemoattractant protein-1 (MCP-1) in plasma and in liver tissue homogenate, due to hepatocyte production, suggesting that Hcy may contribute to chronic inflammation in this organ. In agreement with these results, Ji and Kaplowitz (2004) showed that hyperhomocysteinemia increased mediators of inflammation, such as nuclear factor kappa B (NFκB), interleukin(IL)-1β, IL-6 and IL-8 in liver.

We also evaluated hepatic fibrillar collagen accumulation in hyperhomocysteinemic rats by Picrosirius histochemical staining, and observed an increment in collagen fibers surrounding hepatocytes, as well as in the connective tissue present in portal space and around large vessels, when compared to controls. Our data suggests that Hcy administration mediates an important development of fibrosis in liver of rats. Fibrosis within the liver is a result of an imbalance in a dynamic process of both matrix deposition and degradation. The main cell type responsible for matrix production and accumulation following hepatic damage is activated HSC (Henderson and Forbes, 2008). Robert et al. (2005) studied hyperhomocysteinemic CBS-deficient mice and demonstrated foci of perilobular mononuclear inflammatory infiltrate around the vessels and fibrotic strands enveloping hepatocytes, concomitant with an enhanced expression of pro (alpha) 1 collagen type I and proinflammatory cytokines, as well as activation of HSC. In agreement, Distrutti et al. (2008) recently reported that Hcy could alter HSC function. Activated HSC secretes inflammatory cytokines and up-regulates adhesion molecules, accelerating the recruitment of inflammatory cells (Kisseleva and Brenner, 2007; Friedman, 2008), that could be responsible for the inflammatory infiltration observed in our study. However, from another stand point, hepatic fibrosis might be a direct consequence of oxidative stress and inflammation (Poli, 1993; Poli and Parola, 1997; Marra, 2002; Kershenobich Stalnikowitz and Weissbrod, 2003; Kisseleva and Brenner, 2007; Friedman, 2008) promoted by Hcy administration. Further studies are necessary to clarify the effect of cause and consequence unexplained in our results.

Inversely correlated to fibrosis, macrophages have been shown to be a major source of matrix metalloproteinases (MMP), which degrade scar tissue. Nevertheless, HSC possess an additional mechanism to induce fibrosis, by expression of tissue inhibitors of MMPs (TIMPs) (Henderson and Forbes, 2008). In this context, Hcy could increase TIMP-1 expression and synthesis in liver of CBS-deficient mice or *in vitro* in culture of hepatic cells (HSC and

hepatocytes), leading to fibrosis by inhibition of scar degradation (Torres et al., 1999; Robert et al., 2005).

To further characterize the liver damage provoked by Hcy, we extended our investigation to evaluate glycoproteins/glycogen content. PAS-stained liver slices from Hcy-treated rats showed reduced glycoproteins/glycogen content, when compared to control animals, providing more evidences for Hcy-induced liver injury. Hepatic diseases also could be evaluated by serum markers, as aminotransferases activities. In this regard, we measured ALT and AST activities in plasma and liver tissue (cytosolic and mitochondrial fractions) from chronic hyperhomocysteinemic rats. We did not observe any significant change in aminotransferases activities in neither tissue tested, despite the relevant oxidative and histological alterations found in our study. Conversely, Frelut et al. (2006) recently showed a positive correlation between ALT activity and Hcy plasma levels in obese adolescent girls. Experimental models of hyperhomocysteinemia in rats provide evidence for increased serum ALT and AST activities, associated with ROS production and hepatic lipid peroxidation (Huang et al., 2001; Woo et al., 2006). Recently, Yalçınkaya et al. (2009) have shown that a high methionine diet supplemented for 6 months caused hyperhomocysteinemia, and increased serum ALT and AST levels in rats. We believe that the absence of significantly differences in aminotransferase levels in our study could be due to the short period of treatment with Hcy (22 days).

Taken together, our results presented above qualified the occurrence of oxidative stress, notably reduced antioxidant defenses (enzymatic and non-enzymatic) and increased lipid peroxidation, and inflammatory infiltration in liver of Hcy-treated rats. Both processes have been strongly associated to HSC activation, and consequent fibrosis in liver (Poli, 1993; Poli and Parola, 1997; Marra, 2002; Kershenobich Stalnikowitz and Weissbrod, 2003; Kisseleva and Brenner, 2007; Friedman, 2008). In this context, histological analysis showed that hyperhomocysteinemia prompted to collagen deposition, and reduced glycoproteins/glycogen content in the liver. The present results provide an additional insight into the hepatotoxic mechanisms of Hcy, and may contribute to explain the complex factors involved in liver injury exhibited by hyperhomocysteinemic patients.

Acknowledgments

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Capítulo III

HYPERHOMOCYSTEINEMIA ALTERS CYTOCHROME C OXIDASE ACTIVITY IN PARIETAL CORTEX OF RATS: NEUROPROTECTIVE ROLE OF FOLIC ACID

Cristiane Matté, Vanize Mackedanz, Francieli M. Stefanello, Virgínia C. Rech, Moacir Wajner, Clovis M. D. Wannmacher, Angela T. S. Wyse

Status: A ser submetido

HYPERHOMOCYSTEINEMIA ALTERS CYTOCHROME C OXIDASE ACTIVITY IN PARIETAL CORTEX OF RATS: NEUROPROTECTIVE ROLE OF FOLIC ACID

Cristiane Matté, Vanize Mackedanz, Francieli M. Stefanello, Virgínia C. Rech, Moacir Wajner, Clovis M. D. Wannmacher, Angela T. S. Wyse

Laboratório de Neuroproteção e Doenças Metabólicas, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil.

Address reprint requests to: Dr. Angela T. S. Wyse, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil, Phone: 55 51 3308 5573, Fax: 55 51 3308 5535, E-mail: wyse@ufrgs.br

Abbreviations: ANOVA, analysis of variance; CBS, cystathionine β-synthase; CK, creatine kinase; COX, cytochrome c oxidase; DCIP, dichloroindophenol; Hcy, homocysteine; PMS, phenazine methasulphate; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SPSS, statistical package for the social sciences.

Running title: Folic acid prevents COX alteration Hcy-induced

Abstract

Hyperhomocysteinemia has been identified in neurodegenerative, vascular and metabolic diseases. Homocystinuria, an inborn error of metabolism, is biochemically characterized by cystathionine β-synthase deficiency and homocysteine tissue accumulation. Mitochondrial dysfunction has been reported in patients with vascular and neurodegenerative disorders, and could be related to lipid peroxidation. This study initially investigated the effect of acute and chronic hyperhomocysteinemia on respiratory chain enzymes and creatine kinase activities in parietal cortex of rats. We also evaluated the effect of folic acid treatment on homocysteine-induced alterations. Our results showed that homocysteine acute administration did not alter complex II, succinate dehydrogenase and creatine kinase, but reduced cytochrome c oxidase activity. Conversely, chronic hyperhomocysteinemia increased cytochrome c oxidase activity in parietal cortex of rats. Folic acid prevented such effects, probably by its antioxidant properties. More studies are necessary to evaluate whether folic acid could be used as an adjuvant therapy in hyperhomocysteinemic patients.

Key words: Homocysteine; Folic acid; Cytochrome c oxidase; Lipid peroxidation; Mitochondrial dysfunction; Parietal cortex

1. Introduction

Homocysteine (Hcy), a sulphur containing amino acid, has been associated with a large range of pathologies, such as ischemic stroke and others cerebrovascular disorders (Clarke et al., 1999; Diaz-Arrastia, 2000; Williams et al., 2001; Ullegaddi et al., 2006), neurodegenerative diseases (Mattson et al., 2002; Mattson and Haberman, 2003; Sachdev, 2004), and homocystinuria (Mudd et al., 2001). In this context, we developed in our laboratory an experimental model of hyperhomocysteinemia (Streck et al., 2002), which is the main characteristic of homocystinuria (Mudd et al., 2001). This metabolic disease possesses a high incidence of 1:300,000; and is biochemically characterized by severe deficiency of cystathionine β-synthase (CBS, EC 4.2.1.22), the first enzyme of Hcy transsulfuration pathway (Mudd et al., 2001). Patients affected present signs and symptoms that include mental retardation, seizures, thromboembolism and atherosclerosis (Mudd et al., 2001).

Mitochondria own a central role in the cell life, considering that it be the main site of ATP regeneration. On the other hand, mitochondria are also an important source of reactive oxygen species (ROS), leading to mitochondrial dysfunction (Cadenas and Davies 2000; Nicholls and Budd 2000), which has been associated to neurodegenerative (Beal 2000; Sullivan and Brown 2005; Zeviani and Carelli 2007) and cardiovascular diseases (Chen et al. 2001; Gao et al. 2008). There is a strong body of evidence suggesting that ROS production and concurrent lipid peroxidation, mainly involving cardiolipin, contribute to disrupt the mitochondrial respiratory chain (Muralikrishna Adibhatla and Hatcher, 2006).

Folic acid, a B-vitamin, is the main methyl donor at Hcy remethylation pathway (Finkelstein, 1998; Brosnan et al., 2004), what justify its use as an adjuvant therapy in some hyperhomocysteinemic patients (Walter et al., 1998; Mudd et al., 2001; Ullegaddi et al., 2006; McCaddon, 2006). Another line of evidence indicated that folic acid could act by antioxidant mechanisms (Joshi et al., 2001; Racek et al., 2005; Patro et al., 2006; Au-Yeung et al., 2006). In this context, we have shown that folic acid efficiently prevents Hcy neurotoxic effects, probably acting as an antioxidant (Matté et al., 2006; 2007; in press).

In order to verify the effect of Hcy on energy metabolism in parietal cortex of rats, our first objective in the present study was evaluate the effect of acute and chronic hyperhomocysteinemia on complex II (EC 1.3.5.1), succinate dehydrogenase (SDH, EC 1.3.99.1), cytochrome c oxidase (COX, EC 1.9.3.1), and creatine kinase (CK, EC 2.7.3.2) activities. Next, we investigated whether folic acid treatment could prevent Hcy-induced mitochondrial dysfunction in parietal cortex of rats. We also verify whether the lipid peroxidation participates of the respiratory chain enzymes impair elicited by Hcy acute administration. We used parietal cortex because this cerebral structures is involved in various neurotoxic effects caused by Hcy (Matté et al. 2004; 2006; 2007; 2009).

2. Experimental procedure

2.1. Animals and reagents

One hundred and eighty-two Wistar rats were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da

Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996), and the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental were followed in all experiments. The study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil.

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Drugs administration procedure

2.2.1. Folic acid and homocysteine chronic treatments

Folic acid and $_{D,L}$-Hcy were dissolved in 0.85% NaCl solution (saline) and buffered to pH 7.4. Hcy solution (0.3 - 0.6 μmol/g body weight) was administered subcutaneously twice a day at 8 h interval from 6th to 28th day-of-age (chronic hyperhomocysteinemia). Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Streck et al., 2002), and achieved 123 μM in plasma and 16 μM in parietal cortex of Hcy-treated rats (Matté et al., 2007). Hcy plasma levels are similar to those found in homocystinuric patients (Mudd et al., 2001). Folic acid solution (0.011 μmol/g of weight body) was injected intraperitonealy, once a day, from 6th to 28th day-of-age (Matté et al., 2007). Control animals received saline solution in the same

volumes as those applied to Hcy- and folic acid-treated rats. We administered 1 mL/100 g of weight body in all treatments.

The rats were sacrificed by decapitation without anesthesia twelve hours after the last injection. The brain was quickly removed and parietal cortex was dissected.

2.2.2. Vitamins pretreatment followed by homocysteine acute administration

Folic acid, vitamins E plus C, and $_{D,L}$-Hcy were dissolved in 0.85% NaCl solution (saline) and buffered to pH 7.4. Animals were pretreated daily with an intraperitoneal injection of folic acid (0.011 μmol/g of weight body; Matté et al., 2004), or vitamins E plus C (40 mg/kg and 100 mg/kg of weight body, respectively; Delwing et al., 2007), or saline from the 22th to the 28th day-of-age. Twelve hours after the last injection, the rats received a single subcutaneous injection of Hcy (0.6 μmol/g of weight body; acute hyperhomocysteinemia; Streck et al., 2002) or saline and were sacrificed by decapitation without anesthesia thirty minutes (chemiluminescence assay), one (chemiluminescence assay, creatine kinase, complex II, SDH and COX activities) or twelve hours later (COX activity). We administered 1 mL/100 g of weight body in all treatments. The brain was quickly removed and parietal cortex was dissected.

2.3. Tissue preparation

For cytosolic and mitochondrial fractions isolation to CK activity determination, the parietal cortex was homogenized (1:20 w/v) in ice-cold TRIS-sucrose buffer, pH 7.5, containing 320 mM sucrose, 1 mM EGTA and 10 mM Trizma base. The homogenate was centrifuged at 800 x g for 10 min at 4°C, the

pellet was discarded and the supernatant was centrifuged at 10,000 x g for 15 min at 4°C. The supernatant of the second centrifugation, containing cytosol, was collected for determination of cytosolic CK activity. The pellet, containing mitochondria, was washed twice with the same TRIS-sucrose isotonic buffer, ressuspended in 100 mM $MgSO_4$-Trizma buffer, pH 7.5, for determination of mitochondrial CK activity. Cytosolic and mitochondrial fractions were stored at -70°C when the assay was not carried out immediately. The mitochondrial fraction was frozen and thawed three times immediately before the enzymatic assay to facilitate the interaction between the enzyme and their substrates.

For determination of respiratory chain enzymes activities, the parietal cortex was homogenized (1:10 w/v) in ice-cold SETH buffer, pH 7.4, containing 250 mM sucrose, 2 mM EDTA, 10 mM Trizma base and 50 IU/mL heparin. The homogenates were centrifuged at 800 x g for 10 min at 4°C to discard nuclei and cell debris, the supernatant kept at -70°C until used for enzymes activities determination.

For chemiluminescence, the parietal cortex was homogenized (1:10 w/v) in ice-cold 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 800 x g for 10 min at 4°C, the pellet was discarded and the supernatant was immediately separated and used for the measurements.

2.4. Creatine kinase activity assay

The reaction mixture contained the following final concentrations: 60 mM Tris-HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, and approximately 1 μg protein in a final volume of 0.1 mL. After preincubation at

37℃, the reaction was started by the addition of 0.3 μmol ADP. The reaction was stopped after 10 min by the addition of 1 μmol p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to discount chemical hydrolysis of phosphocreatine and the amount of creatine already present in mitochondrial and cytosolic fractions. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 2% α-naphtol and 0.1 mL 0.05% diacetyl in a final volume of 1 mL, and read after 20 min at 540 nm. Results were expressed as μmol creatine/min.mg protein.

2.5. Complex II and succinate dehydrogenase activities assay

Complex II (systematic name: succinate:ubiquinone oxidoreductase) and SDH (systematic name: succinate:acceptor oxidoreductase) were measured in parietal cortex homogenates following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as reference wavelength (ε = 19.1 mM^{-1} cm^{-1}) in the presence of phenazine methasulphate (PMS), according to Fischer et al. (1985). The reaction mixture consisting of 40 mM potassium phosphate buffer, pH 7.4, 16 mM succinate and 8 μM DCIP were preincubated with 40-80 μg protein at 30℃ for 20 min. Subsequently, for complex II activity determination, we added 4 mM sodium azide and 7 μM rotenone, the reaction was initiated by addition of 40 μM DCIP and was monitored for 5 min. The activity of SDH was accessed in the same incubation medium by addition of 1 mM PMS and monitored for 5 min. Complex II and SDH activities were expressed as nmol/min.mg protein.

2.6. Cytochrome c oxidase activity assay

COX (systematic name: ferrocytochrome-*c*:oxygen oxidoreductase) activity was measured according to Rustin et al. (1994), by following the decrease in absorbance due to oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength (ε = 19.1 mM^{-1} cm^{-1}). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2-4 μg protein, and the reaction was initiated by the addition of 0.7 μg reduced cytochrome *c*. COX activity was measured at 25°C for 10 min, and was expressed as nmol/min.mg protein.

2.7. Chemiluminescence assay

Samples were assayed for chemiluminescence in accord to Lissi et al. (1986), in a dark room using a beta liquid scintillation spectrometer Tri-Carb 2100TR. Incubation flasks contained 3.5 mL of the 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The background chemiluminescence was measured for 5 min. An aliquot of 0.5 mL of supernatant was added and chemiluminescence was measured for 10 min at room temperature. The background chemiluminescence was subtracted from the total value. Chemiluminescence was calculated as cps/mg protein.

2.8. Protein determination

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

2.9. Statistical analysis

Data were analyzed by the Student's *t* test, or one-way analysis of variance (ANOVA) followed by post-hoc Duncan's multiple range test when *F* value was significant, as indicated. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. Differences were considered statistically significant if $p<0.05$.

3. Results

3.1. Effect of acute and chronic hyperhomocysteinemia on creatine kinase and respiratory chain enzymes activities in parietal cortex of rats

Table 1 shows that acute Hcy administration did not alter CK activity in cytosolic [$t(10)= -0.383$; $p>0.05$] and mitochondrial [$t(10)= -1.094$; $p>0.05$] fractions in parietal cortex of rats sacrificed one hour after injection. In order to investigate if repetitive Hcy administration might alter CK activity, we evaluated the effect of chronic hyperhomocysteinemia in CK activity. Table 1 also shows that chronic Hcy administration did not alter CK activity in cytosolic [$t(9)= -0.387$; $p>0.05$] and mitochondrial [$t(6)= 0.885$; $p>0.05$] fractions of parietal cortex of rats.

Next we evaluated the effect of acute Hcy administration on complex II, SDH, and COX activities in parietal cortex of rats sacrificed one hour after administration. As can be observed in Figure 1, complex II [A; $t(10)= 0.138$; $p>0.05$] and SDH [B; $t(10)= -0.152$; $p>0.05$] activities were not altered by Hcy administration. In contrast, COX activity was significantly reduced [Figure 1C; $t(12)= 3.12$; $p<0.01$] in parietal cortex of acute hyperhomocysteinemic rats.

Conversely, animals sacrificed twelve hours after the last injection of Hcy did not present any alteration on COX activity in parietal cortex [Control = 313 ± 37; Hcy = 316 ± 20; $t(8)= -0.137$; $p>0.05$].

Likewise acute hyperhomocysteinemia, chronic Hcy administration did not alter complex II [A; $t(10)= -0.486$; $p>0.05$] and SDH [B; $t(10)= -0.025$; $p>0.05$] activities in parietal cortex of rats (Figure 2). Opposing our expectations, chronic hyperhomocysteinemia significantly increased COX activity in parietal cortex of rats [Figure 2C; $t(12)= -4.79$; $p<0.001$]. We also evaluated the effect of chronic hyperhomocysteinemia on same respiratory chain enzymes in parietal cortex of rats sacrificed one hour after the last Hcy administration, and found similar results to those described to animals sacrificed twelve hours after the last Hcy injection (data not shown).

3.2. Effect of folic acid and/or vitamins E plus C pretreatment on biochemical alterations elicited by hyperhomocysteinemia in parietal cortex of rats

Figure 3 shows that folic acid [A; $F(3,24)= 7.30$; $p<0.01$] and vitamins E plus C [B; $F(3,17)= 4.66$; $p<0.05$] prevented COX activity inhibition in parietal cortex of acute hyperhomocysteinemic rats. We also investigated whether lipid peroxidation was involved on Hcy acute mechanism of neurotoxicity verified in parietal cortex of rats, and if folic acid could act as an antioxidant in our experimental model. Figure 4 shows that pretreatment with folic acid prevented the increase in chemiluminescence caused by Hcy acute administration in rats sacrificed 30 [A; $F(3,20)= 3.62$; $p<0.05$] or 60 minutes afterward [B; $F(3,20)= 3.67$; $p<0.05$]. Folic acid pretreatment *per se* did not alter chemiluminescence when compared to control group.

Finally, we evaluated whether folic acid concurrent administration might prevent Hcy chronic effect on COX activity in parietal cortex of rats. Figure 5 shows that folic acid *per se* did not alter COX activity, but prevented the increase of COX activity when administered concomitantly with Hcy from their 6^{th} to their 28^{th} day-of-age [$F(3,24)= 6.71$; $p<0.01$].

4. Discussion

In the present study, firstly we investigated the effect of Hcy acute administration on mitochondrial enzymes activities. Results showed that Hcy acute administration inhibited COX activity, and did not alter SDH, complex II and CK activities in parietal cortex of rats sacrificed one hour after Hcy injection. Twelve hours after Hcy administration, COX activity returned to control levels.

Our results are in agreement with Linnebank et al. (2006), who reported that Hcy *in vitro* altered COX activity and induced ROS generation and apoptotic cell death in rat and human neurons. COX, a membrane-embedded enzyme complex, appear to be more susceptible to oxidative modifications than others mitochondrial complexes, moreover it could be impaired by lipid peroxidation and cardiolipin loss in brain (Delgado et al., 2006; Sen et al., 2006; Pope et al., 2008). In addition, Gao et al. (2008) reported that COX inhibition could be followed by ATP reduction and by ROS production. In this context, we previous showed that chronic Hcy administration provokes lipid peroxidation, DNA damage, and reduced antioxidant defenses in parietal cortex of rats (Matté et al. 2007; 2009). However, in literature there is not a consensus about the

relationship, of cause or consequence, between ROS production and COX inhibition.

Next, we performed a pretreatment with folic acid for one week to evaluate whether this B vitamin might prevent Hcy effects. We found that folic acid prevented COX inhibition in parietal cortex of acute hyperhomocysteinemic rats. Evidences suggest that folic acid possess antioxidant properties, modulating ROS generation by Fenton reaction (Patro et al., 2006), and/or directly scavenging ROS, such as $OH^{\bullet}$ (Joshi et al., 2001). With the purpose of establish that folic acid has antioxidant properties in our animal model, we investigated the effect of pretreatment with this vitamin in chemiluminescence emitted by ROS during lipid peroxidation elicited by Hcy administration. We observed that folic acid prevented the increase of chemiluminescence in parietal cortex of hyperhomocysteinemic rats. These results are in agreement with our previous results showing that folic acid chronic administration decreases lipid peroxidation, measured by thiobarbituric acid-reactive substances assay (Matté et al., 2007). Recently, we also observed that DNA damage and the reduction of antioxidant defenses in parietal cortex of rats subjected to hyperhomocysteinemia were prevented by folate (Matté et al. 2009).

In order to verify whether oxidative stress is involved in Hcy-induced COX inhibition, we performed studies based on pretreatment with vitamins E plus C, a classical antioxidant combination (Wyse et al., 2002; Reis et al., 2002; Martin et al., 2002; Dewing et al., 2007). Results showed that vitamins E plus C prevented COX inhibition in parietal cortex provoked by acute hyperhomocysteinemia. Taken together these results suggest that COX was

inhibited by acute Hcy administration by an oxidative stress-related mechanism, probably lipid peroxidation. However, we could not define if lipid peroxidation was a cause and/or a consequence from alterations in mitochondrial homeostasis.

We also evaluated the effect of chronic Hcy administration on respiratory chain enzymes (SDH, complex II, and COX) and CK in parietal cortex of rats. We observed that chronic hyperhomocysteinemia increased COX activity and did not alter the activities of SDH and complex II. We previous showed that chronic Hcy administration induces oxidative stress, increases lipid peroxidation (Matté et al., 2007), provokes DNA damage, and reduced antioxidant defenses in parietal cortex of rats (Matté et al., 2009). Corroborating with this idea, Huertas et al. (1992) showed that oxidative stress was able to induce a significant increase at COX levels in rat heart. In agree with our data, Austin et al. (1998) showed that Hcy increased the expression of mitochondrial electron transport chain gene COX III/ATPase 6,8 in a human megakaryocytic cell line after 18 hours of incubation, in a concentration- and time-dependent manner.

Neurodegenerative diseases have been associated to mitochondrial alterations (Beal, 2000; Sullivan and Brown, 2005; Zeviani and Carelli, 2007) and possess a well studied role of hyperhomocysteinemia in their pathogenesis (Mattson et al., 2002; Mattson and Haberman, 2003; Sachdev, 2004). In this context, a relevant study performed with Alzheimer's patients reported that brain samples present increased levels of COX (Manczak et al., 2004). Kadenbach et al. (2004) also showed that increase in COX activity was followed by ROS generation and could result in neurodegeneration. In our study we verified that chronic Hcy administration induced an increase in COX activity, possible by

oxidative modulation (Matté et al., 2007; in press), however we could not discard other mechanisms by which Hcy induces activation of COX activity in parietal cortex, such as increase in the expression of this enzyme.

Finally, we investigated whether folic acid concurrent treatment could prevent the COX increases observed in parietal cortex of hyperhomocysteinemic rats. Folic acid prevented such effect possibly for its antioxidant role since previously we showed that this vitamin, in the same dose, did not alter elevated plasma Hcy levels, but prevented oxidative damage caused by chronic Hcy administration (Matté et al., 2007; 2009). In addition, folate deprivation resulted in increased Hcy plasma levels, and was followed by oxidative damage, verified by 8-hydroxydeoxyguanosine content, and by enhanced expression of mRNA for nuclear-encoded COX subunits in liver (Chou et al., 2007).

It has been shown that Hcy and its metabolites are related to epilepsy and energy metabolism deficit (Kubová et al. 1995; Mares et al. 1997; Streck et al. 2003). In this context, Hcy impairs energy metabolism in hippocampus (Streck et al. 2003) and homocysteic acid, a metabolite of Hcy, decreases complex I activity in cerebral cortex of rats probably by oxidative modifications (Folbergrová et al. 2007). In addition, seizures induced by Hcy thiolactone causes alterations in neurons, electroencephalographic recordings, and in behavioral responses (Stanojlovic et al. 2009), as well as reduces phosphocreatine content in cerebral cortex of rats (Folbergrová 1993).

Our results together with previous reports suggest that Hcy induced lipid peroxidation and modulated COX activity by two different ways. Hcy short-term effect, verified in acute hyperhomocysteinemia, was characterized by inhibition

of COX activity; conversely, long-term effect, represented by chronic hyperhomocysteinemia, was marked by activation of COX in parietal cortex of rats. Folic acid treatment efficiently prevented COX alterations and lipid peroxidation in parietal cortex of hyperhomocysteinemic rats. Whether our results might be extrapolated to human condition, we suggest the evaluation of folic acid as an adjuvant therapy, in order to diminish mitochondrial dysfunction related to neurological disorders observed in hyperhomocysteinemic patients.

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Figure 1. Effect of homocysteine acute administration on complex II (A), succinate dehydrogenase (B) and cytochrome c oxidase (C) activities in parietal cortex of rats (sacrificed 1 h later). Data are expressed as mean ± S.D. for 5-7 independent experiments (animals) performed in duplicate. Different from control, **p<0.01 (Student's t test). Hcy: homocysteine

Figure 2. Effect of homocysteine chronic administration on complex II (A), succinate dehydrogenase (B) and cytochrome c oxidase (C) activities in parietal cortex of rats. Data are expressed as mean ± S.D. for 6-7 independent experiments (animals) performed in duplicate. Different from control, ***p<0.001 (Student's t test). Hcy: homocysteine

Figure 3. Effect of folic acid (A) and vitamins E plus C (B) pretreatment on cytochrome c oxidase activity inhibition in parietal cortex of rats subjected to acute hyperhomocysteinemia (sacrificed 1 h later). Data are expressed as mean ± S.D. for 5-7 independent experiments (animals) performed in duplicate. Different from control, *p<0.05; **p<0.01 (one way ANOVA followed by Duncan's multiple range test). Hcy: homocysteine

Figure 4. Effect of folic acid pretreatment on chemiluminescence increases in parietal cortex of rats subjected to acute hyperhomocysteinemia and sacrificed 30 (A) or 60 (B) minutes after homocysteine injection. Data are expressed as mean ± S.D. for 6 independent experiments (animals) performed in duplicate. Different from control, *p<0.05 (one way ANOVA followed by Duncan's multiple range test). Hcy: homocysteine

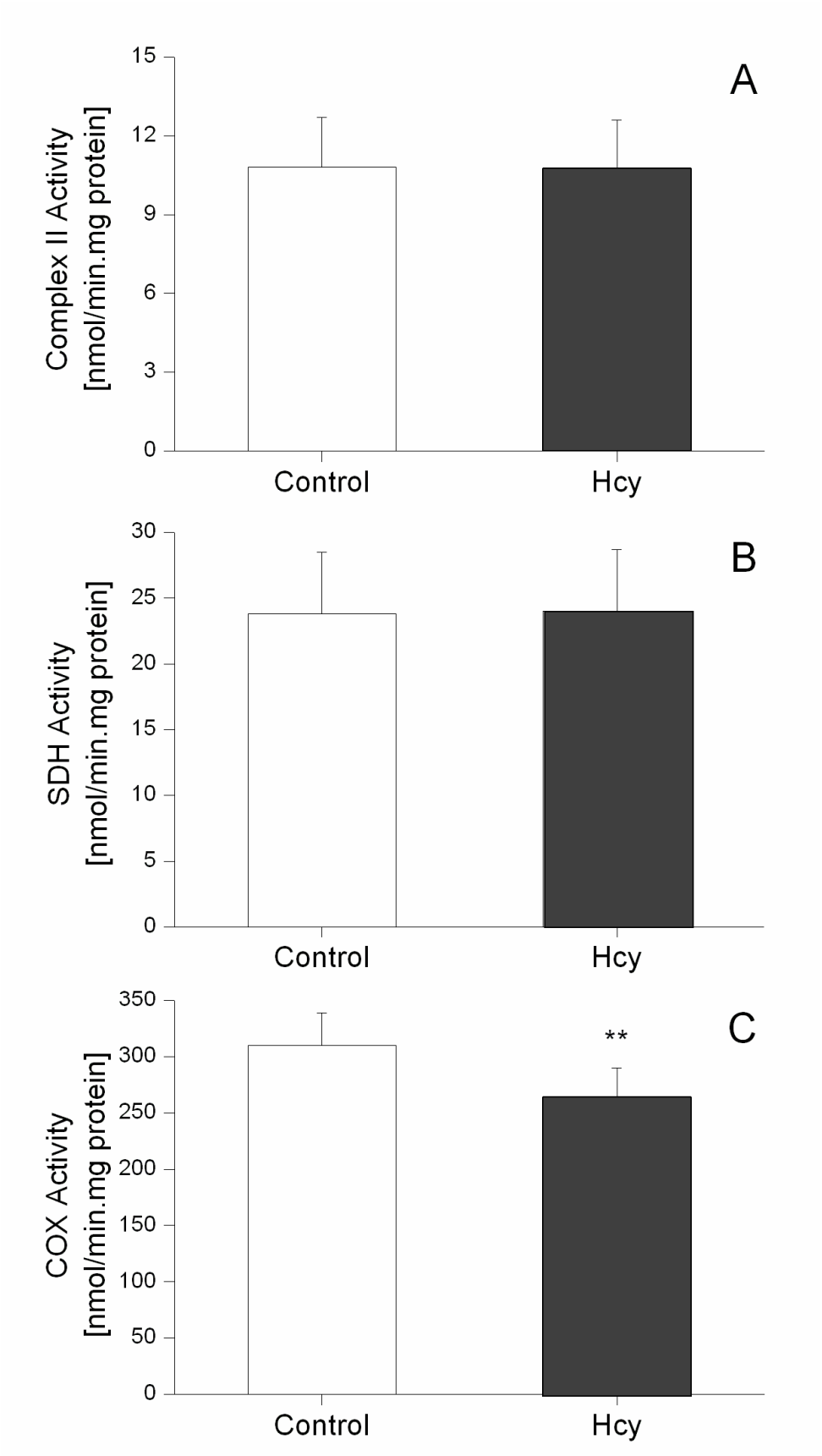
Figure 5. Effect of folic acid chronic administration on cytochrome c oxidase activity increases in parietal cortex of rats subjected to chronic hyperhomocysteinemia. Data are expressed as mean ± S.D. for 7 independent experiments (animals) performed in duplicate. Different from control, **$p<0.01$ (one way ANOVA followed by Duncan's multiple range test). Hcy: homocysteine

Table 1 Effect of homocysteine acute and chronic administrations on creatine kinase activity in parietal cortex of rats

	Creatine Kinase Activity [% of control]	
	Cytosolic fraction	Mitochondrial fraction
Acute hyperhomocysteinemia		
Control	100 ± 25.3	100 ± 22.1
Homocysteine	100 ± 36.9	118 ± 28.8
Chronic hyperhomocysteinemia		
Control	100 ± 18.9	100 ± 31.6
Homocysteine	103 ± 10.9	85.5 ± 11.2

Data are expressed as mean ± S.D. for 4-6 animals, performed in triplicate. There was no significant difference between groups (Student's t test)

Figure 1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Complex II Activity
[nmol/min.mg protein]
15
12
9
6
3
0
Control
Hcy
B
SDH Activity
[nmol/min.mg protein]
30
25
20
15
10
5
0
Control
Hcy
C
COX Activity
[nmol/min.mg protein]
350
300
250
200
150
100
50
0
**
Control
Hcy

Figure 2

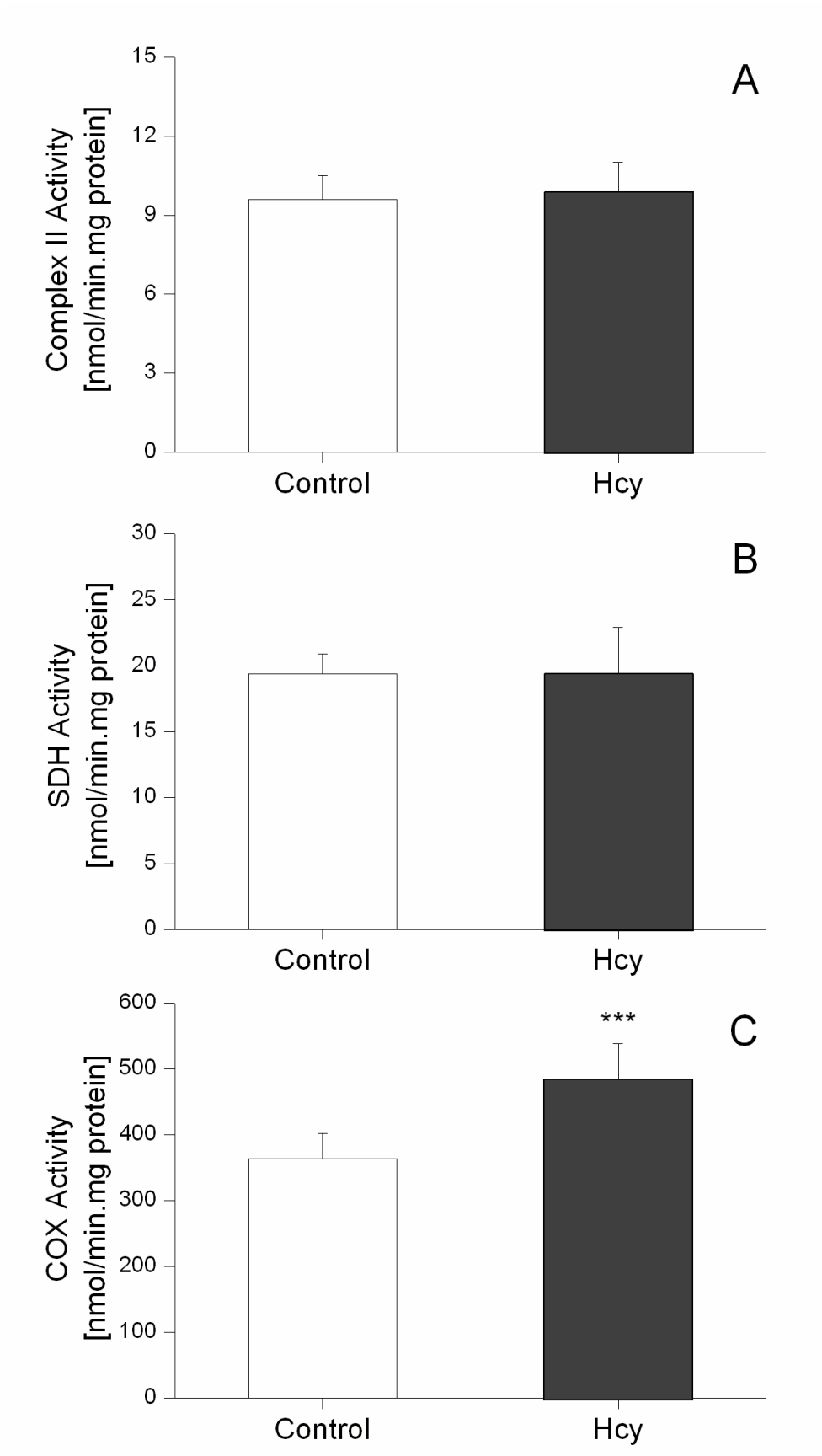

A
Complex II Activity
[nmol/min.mg protein]
15
12
9
6
3
0
Control
Hcy
B
SDH Activity
[nmol/min.mg protein]
30
25
20
15
10
5
0
Control
Hcy
C
***
COX Activity
[nmol/min.mg protein]
600
500
400
300
200
100
0
Control
Hcy

Figure 3

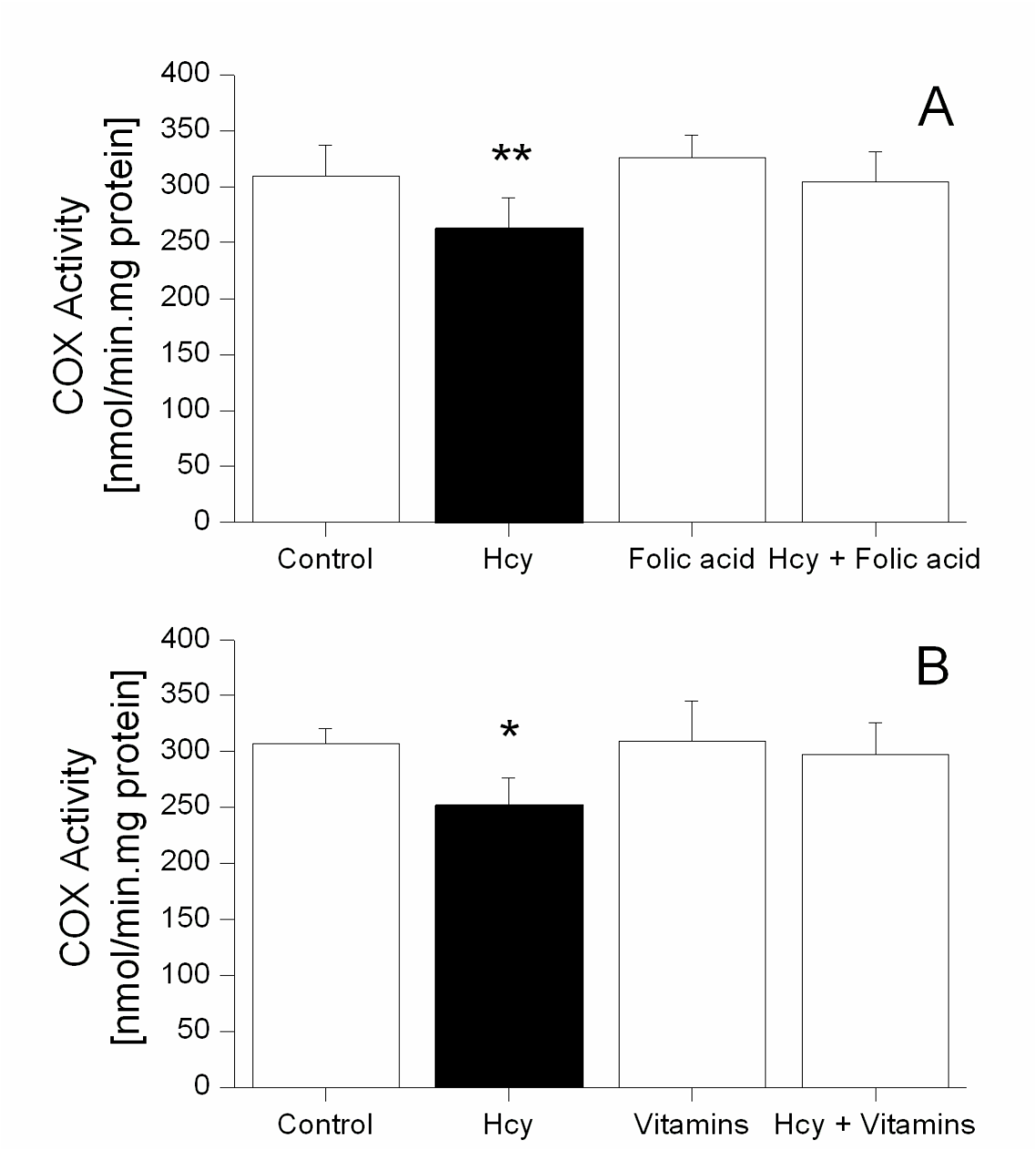

A
COX Activity
[nmol/min.mg protein]
400
350
300
250
200
150
100
50
0
**
Control
Hcy
Folic acid
Hcy + Folic acid
B
COX Activity
[nmol/min.mg protein]
400
350
300
250
200
150
100
50
0
*
Control
Hcy
Vitamins
Hcy + Vitamins

Figure 4

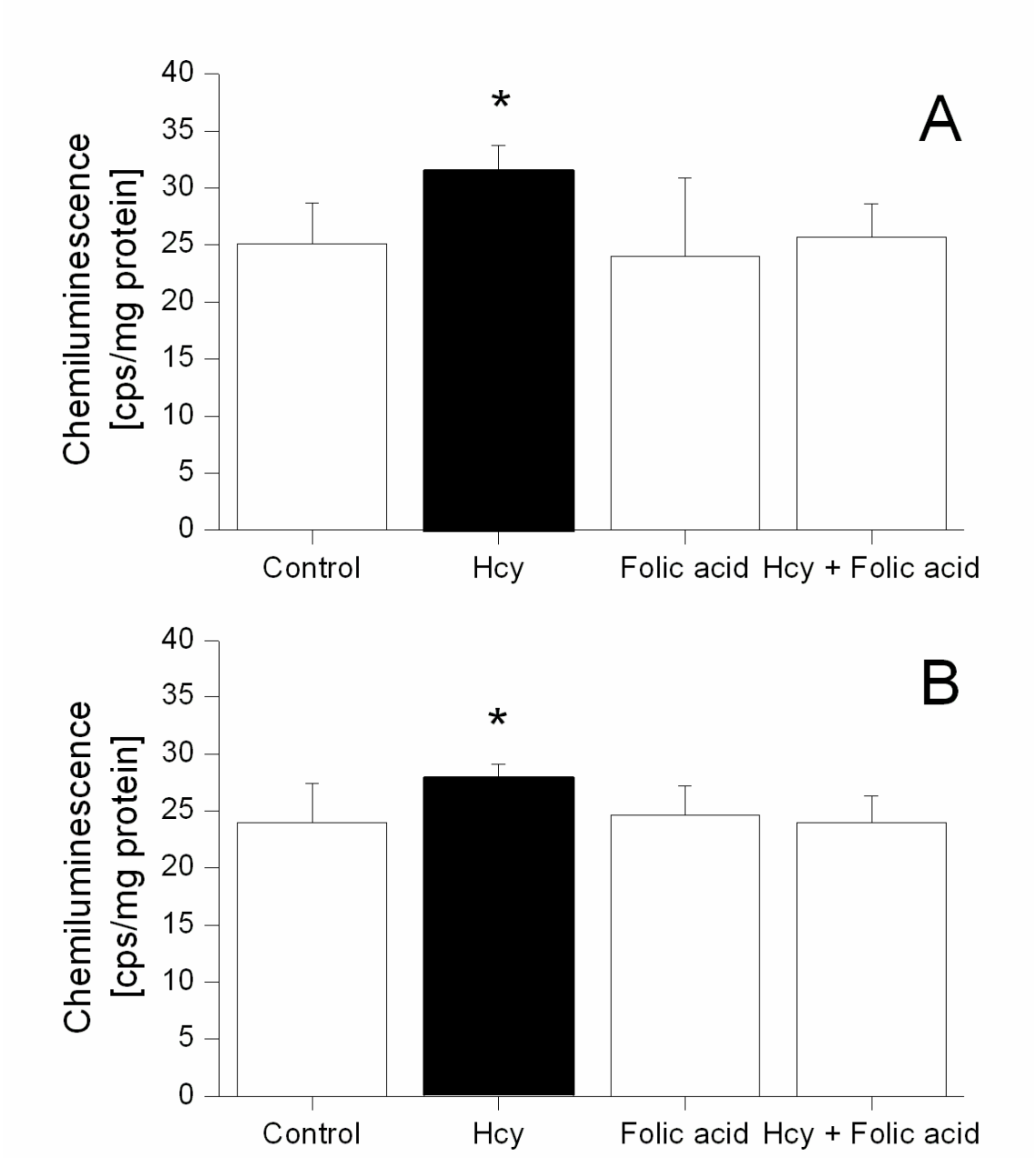
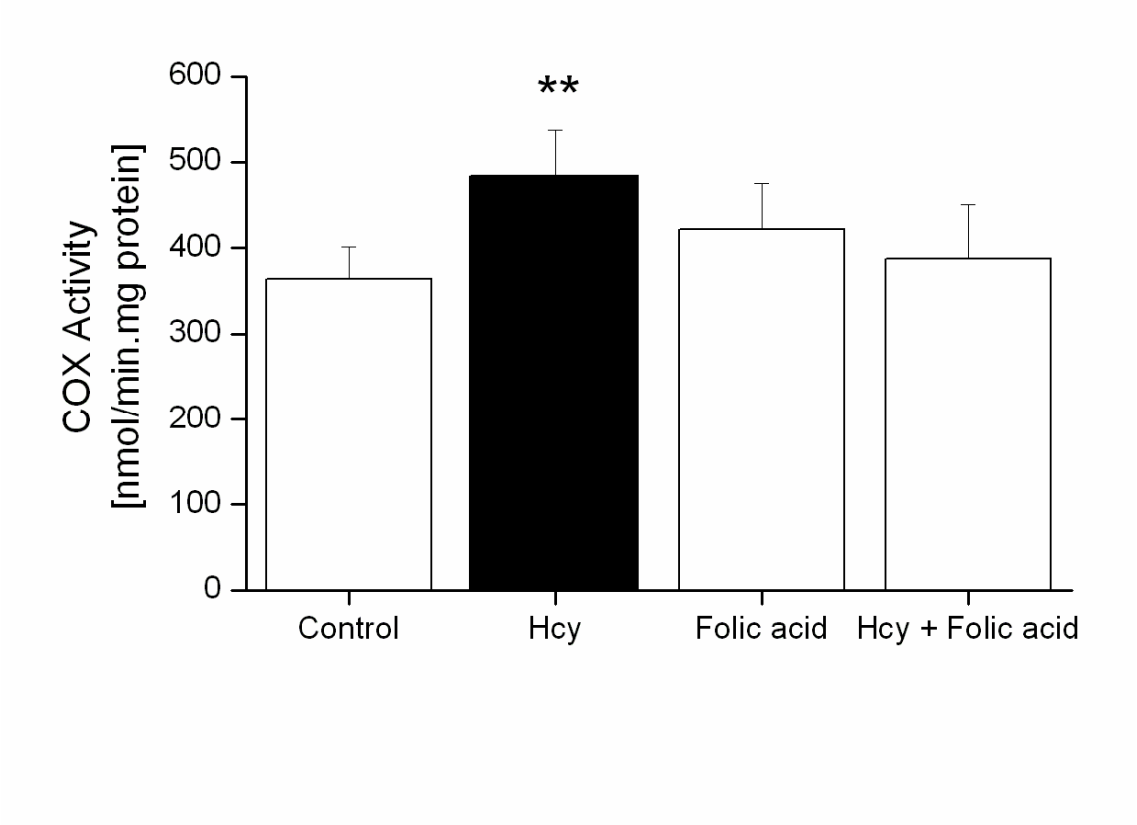

A
B
Chemiluminescence [cps/mg protein]
40
35
30
25
20
15
10
5
0
*
Control
Hcy
Folic acid
Hcy + Folic acid

Figure 5


COX Activity
[nmol/min.mg protein]
600
500
400
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200
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**
Control
Hcy
Folic acid
Hcy + Folic acid

Capítulo IV

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Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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ABSTRACT

In the present study we evaluated the effect of acute and chronic homocysteine administrations on glutamate uptake in parietal cortex of rats. The immunocontent of glial glutamate transporter (GLAST) and sodium-dependent glutamate/aspartate transporter (GLT-1) in the same cerebral structure was also investigated. For acute treatment, neonate or young rats received a single injection of homocysteine or saline (control) and were sacrificed 1, 8, 12 h, 7 or 30 days later. For chronic treatment, homocysteine was administered to rats twice a day at 8 h interval from their 6th to their 28th days old; controls and treated rats were sacrificed 12 h, 1, 7 or 30 days after the last injection. Results show that acute hyperhomocysteinemia caused a reduction on glutamate uptake in parietal cortex of neonate and young rats, and that 12 h after homocysteine administration the glutamate uptake returned to normal levels in young rats, but not in neonate. Chronic hyperhomocysteinemia reduced glutamate uptake, and GLAST and GLT-1 immunocontent. According to our results, it seems reasonable to postulate that the reduction on glutamate uptake in cerebral cortex of rats caused by homocysteine may be mediated by the reduction of GLAST and GLT-1 immunocontent, leading to increased extracellular glutamate concentrations, promoting excitotoxicity.

1. Introduction

Homocysteine (Hcy), a methionine-derived sulphur amino acid, has been associated with several disorders that affect the CNS, such as epilepsy (Sachdev, 2004; Herrmann et al., 2007), stroke (Obeid et al., 2007), neurodegenerative (Clarke et al., 1998; Mattson et al., 2002) and neuropsychiatric diseases (Diaz-Arrastia, 2000; Bottiglieri, 2005), as well as inborn errors of metabolism (Mudd et al., 2001). Homocystinuria is biochemically characterized by cystathionine β-synthase (E.C. 4.2.1.22) deficiency, resulting in accumulation of Hcy and its metabolites in the body. Clinically, affected patients present pathological manifestations in several organs, mainly on vascular and central nervous systems (CNS), including mental retardation, psychiatric disturbances, seizures, thromboembolism, and cardiovascular complications (Mudd et al., 2001).

Glutamatergic excitotoxicity appears to be associated with brain damage caused by Hcy. In this context, previous reports suggest that Hcy induces neurodegeneration by NMDA receptor overstimulation (Lipton et al., 1997; Jara-Prado et al., 2003; Zieminska et al., 2003; Zieminska and Lazarewicz, 2006; Poddar and Paul, 2009). Although the glutamatergic excitotoxicity and the neurodegeneration have been associated with overstimulation of postsynaptic receptors, the glutamate transporters have shown a relevant role on physiopathology of these diseases (Sheldon and Robinson, 2007). After signaling action on glutamate receptors, this excitatory amino acid is removed from extracellular fluid, in order to maintain low synaptic and extrasynaptic glutamate concentrations. In this context, the main excitatory amino acid transporters are GLAST/excitatory amino acid transporter (EAAT1) and GLT-1/EAAT2 found predominantly in glial cells (Rothstein et al., 1994; Danbolt, 2001; Maragakis and Rothstein, 2004), although GLT-1 and GLAST have been shown also in neurons (Mennerick et al., 1998; Plachez et al., 2000); followed by excitatory amino acid carrier (EAAC)/EAAT3 present in glial cells and post-synaptically in neurons, EAAT4 found in cerebellar Purkinje cells, and EAAT5 in retina (Rothstein et al., 1994; Danbolt, 2001; Maragakis and Rothstein, 2004).

Considering the increasing relevance of glutamatergic system on neurodegeneration and its correlation with hyperhomocysteinemia, the main objective of the present study was to investigate the effect of acute and chronic Hcy administrations on glutamate uptake in parietal cortex of rats throughout their CNS development. We also evaluated the immunocontent of GLAST and GLT-1 in parietal cortex of rats subjected to chronic hyperhomocysteinemia. Parietal cortex was selected because patients presenting

* Corresponding author at: Laboratório de Neuroproteção e Doenças Metabólicas, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil.
Tel.: +55 51 3308 5573, fax: +55 51 3308 5535.
E-mail address: wyse@ufrgs.br (Angela T.S. Wyse).

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hyperhomocysteinemia exhibit cortical atrophy (Sachdev, 2005), moreover we have shown that Hcy elicits several neurotoxic effects in this cerebral structure (Matté et al., 2006, 2007, 2009).

2. Experimental procedures

2.1. Animals and reagents

One hundred and fifty-nine male Wistar rats (6 or 29 days-of-age) were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant room temperature (22 ± 1 °C). Rats had free access to a 20% (w/w) protein commercial chow and water. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996), and the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental were followed in all experiments. The study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil.

L-[2,3-3H] glutamate (specific activity 30 Ci/mmol) that was purchased from Amersham International, UK. Protease inhibitors were obtained from Roche Molecular Biochemicals. The antibodies used were described in the text. The other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Drug administration procedure

2.2.1. Acute homocysteine administration

D,L-Hcy was dissolved in 0.85% NaCl solution (saline) and buffered to pH 7.4. The rats received a single subcutaneous injection of Hcy, 0.3 or 0.6 μmol/g of weight body given to 6 days-of-age or 29 days-of-age rats, respectively. Hcy crosses the blood brain barrier and presents a peak in the cerebrum 15 min after subcutaneous injection (Streck et al., 2002). In addition, we showed that Hcy concentration was increased also in the parietal cortex, 1 h after subcutaneous administration (Matté et al., 2007). Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. The animals were sacrificed by decapitation without anesthesia 1, 8, 12 h, 7 or 30 days after the injection, as indicated for each experiment. The brain was quickly removed and parietal cortex was dissected.

2.2.2. Chronic homocysteine administration

D,L-Hcy was dissolved in 0.85% NaCl solution (saline) and buffered to pH 7.4. Hcy solution (0.3–0.6 μmol/g body weight) was administered subcutaneously twice a day at 8 h interval from their 6th to their 28th days old. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Streck et al., 2002). Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those found in homocystinuric patients (Mudd et al., 2001). Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. The rats were sacrificed by decapitation without anesthesia 12 h, 1, 7, or 30 days after the last injection. The brain was quickly removed and parietal cortex was dissected.

2.3. Glutamate uptake assay

Glutamate uptake was performed according to a previous report (Delwing et al., 2007). Parietal cortex was cut into 400 μm thick slices with a McIlwain chopper. For each animal, nine cortical slices (6 for total and 3 for sodium-independent uptake) were transferred to 24-well dishes containing 0.5 mL of Hank's balanced salt solution (HBSS), which contains (mM): 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 1.11 glucose, pH 7.2, 35 °C. For total uptake, the slices were preincubated at 35 °C for 15 min. The uptake assay was assessed by adding 20 μL of a solution containing 0.33 μCi/mL L-[2,3-3 H] glutamate with 100 μM unlabeled glutamate at 35 °C. Incubation was stopped after 7 min by two washes with 1 mL ice-cold HBSS immediately followed by addition of 0.5 M NaOH. Aliquots of lysates were taken for determination of intracellular content of L-[2,3-3 H] glutamate by scintillation counting. Sodium-independent uptake was determined by using an ice-cold (4 °C) HBSS containing N-methyl-D-glucamine instead of sodium chloride. The results were subtracted from the total uptake to obtain the sodium-dependent uptake, and were calculated as nmol of glutamate/(mg protein min).

2.4. GLAST and GLT-1 immunocontent assay

For western blot analysis, parietal cortex was homogenized in ice-cold lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% SDS and a cocktail of protease inhibitors (Roche Molecular Biochemicals). Aliquots were taken for protein determination and β-mercaptoethanol was added to a final concentration of 5%. Protein samples (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, following transfer to nitrocellulose membranes. Membranes were blocked for 60 min with 5% powdered milk in tween-Triz-buffered saline (M-T-TBS) and further incubated overnight at 4 °C with the appropriate primary antibody dissolved in M-T-TBS. The primary antibody used was anti-GLAST and anti-GLT-1 (both 1:2000, rabbit polyclonal), that were kindly provided by Dr. D. Pow, University of Newcastle, Australia. After washing, the membranes were incubated for 2 h with anti-rabbit IgG peroxidase-conjugated (1:1000, Amersham plc). Immunoreactive bands were revealed by an enhanced chemiluminescence kit (ECL, Amersham plc) and detected using X-ray films. The immunoblot films were scanned and the digitalized images analyzed with the Optiquant software (Packard Instrument). The same blots were re-probed with β-actin antibody (1:2000, mouse monoclonal; catalog A 5316, Sigma) as an internal control.

2.5. Protein determination

Protein concentration was measured by the method of Peterson (1977), using bovine serum albumin as standard.

2.6. Statistical analysis

Data were expressed as percent of control, however were analyzed as original values expressed as nmol/(mg protein min). One-way analysis of variance (ANOVA), followed by Duncan's test, was used to analyze data from glutamate uptake assays. Student's *t*-test was used to evaluate data from GLAST and GLT-1 immunocontent. 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. Homocysteine administration reduces glutamate uptake in parietal cortex of rats

The classical homocystinuria is the metabolic disease where the high Hcy plasma levels occur, reaching up to 500 μM (Mudd et al., 2001). In this regard, we developed a chemically induced experimental model of hyperhomocysteinemia in rats, by daily subcutaneous Hcy administration (Streck et al., 2002), where Hcy plasma levels achieved are similar to those found in homocystinuric patients (Mudd et al., 2001).

Firstly, we investigated the effect of acute hyperhomocysteinemia on glutamate uptake in slices of cerebral cortex of neonate and young rats. Fig. 1 shows that a single Hcy administration performed in 6-day-old rats is able to inhibit the glutamate uptake measured 1 and 12 h, 7 and 30 days after the injection [$F(4,26) = 13.60$; $p < 0.001$], with a maximum inhibition of 59% observed 7 days after the injection. Fig. 2 shows that a single administration of Hcy to young rats (29-day-old) provoked a significant reduction in glutamate uptake at 1 and 8 h after

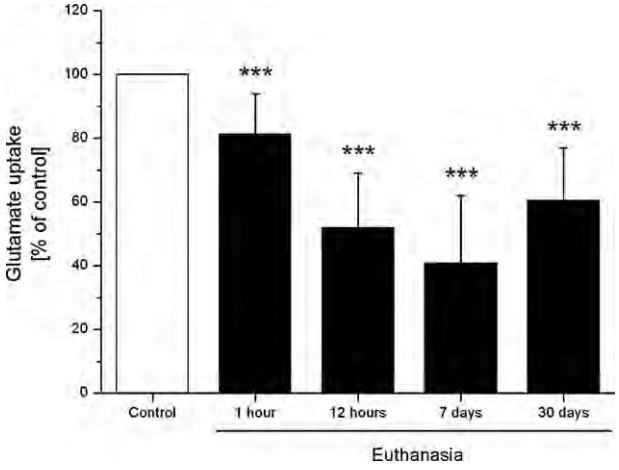

Fig. 1. Effect of acute hyperhomocysteinemia on glutamate uptake in slices of parietal cortex from 6 days-of-age rats euthanized 1, 12 h, 7, or 30 days after homocysteine injection. Results calculated as nmol/(mg protein min) are expressed in % of control as mean ± S.D. for six to seven animals in each group. Different from control, ***$p < 0.001$ (one-way ANOVA followed by Duncan's test).

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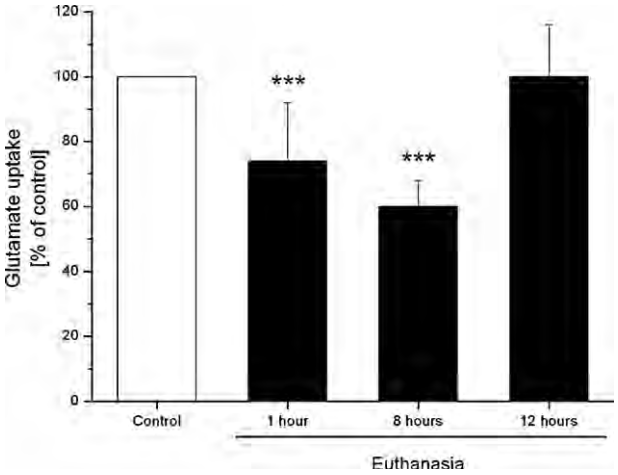

Fig. 2. Effect of acute hyperhomocysteinemia on glutamate uptake in slices of parietal cortex from 29 days-of-age rats euthanized 1, 8, or 12 h after homocysteine injection. Results calculated as nmol/(mg protein min) are expressed in % of control as mean ± S.D. for six animals in each group. Different from control, $^{***}p < 0.001$ (one-way ANOVA followed by Duncan's test).

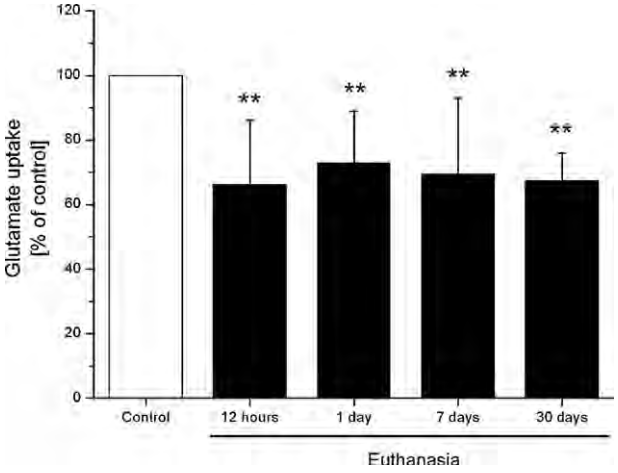

Fig. 3. Effect of chronic hyperhomocysteinemia on glutamate uptake in slices of parietal cortex from rats euthanized 12 h, 1, 7, or 30 days after the last homocysteine injection. Results calculated as nmol/(mg protein min) are expressed in % of control as mean ± S.D. for five to six animals in each group. Different from control, $^{**}p < 0.01$ (one-way ANOVA followed by Duncan's test).

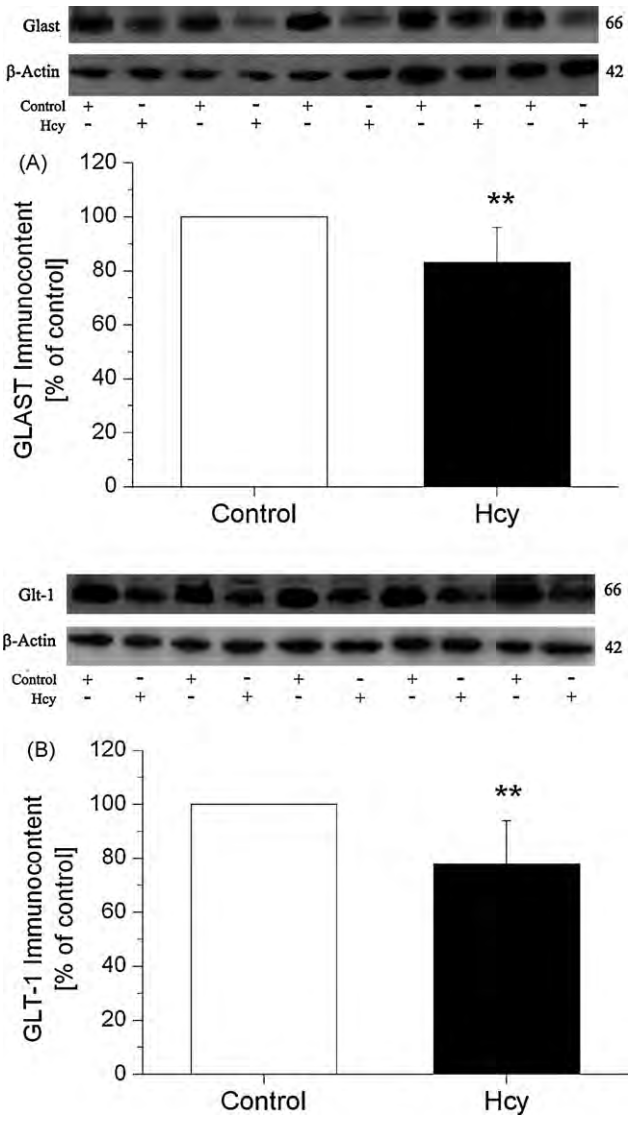

Fig. 4. Effect of chronic hyperhomocysteinemia on glutamate transporters immunocontent, GLAST (A) and GLT-1 (B), in parietal cortex of rats. Results are expressed as mean ± S.D. for six to seven animals in each group. Different from control, $^{**}p < 0.01$ (Student's *t*-test). Hcy: homocysteine.

injection [$F(3,20) = 13.75$; $p < 0.001$], with a maximum inhibition of 40% observed 8 h after injection. However, 12 h after Hcy administration, glutamate uptake returned to control levels in parietal cortex of 29-day-old rats subjected to acute Hcy administration.

We also evaluated the effect of chronic hyperhomocysteinemia on glutamate uptake in slices from parietal cortex of rats. Fig. 3 shows that chronic Hcy administration reduced approximately by 30% the uptake of glutamate [$F(4,24) = 4.433$; $p < 0.01$] at 12 h, 1, 7, and 30 days after the last Hcy injection.

3.2. Chronic hyperhomocysteinemia decreases GLAST and GLT-1 immunocontent in parietal cortex of rats

Glial glutamate transporters GLAST and GLT-1 are proposed to account for the majority of extracellular glutamate uptake. We found a significant reduction on GLAST [$t(12) = 3.339$; $p < 0.01$] and GLT-1 immunocontent [$t(11) = 3.684$; $p < 0.01$] in parietal cortex of rats subjected to a chronic Hcy administration, and sacrificed 12 h after the last injection (Fig. 4).

4. Discussion

Moderate Hcy plasma levels are associated with epilepsy (Sachdev, 2004; Herrmann et al., 2007), neurodegenerative (Clarke et al., 1998; Mattson et al., 2002) and neuropsychiatric diseases (Diaz-Arrastia, 2000; Bottiglieri, 2005). In the present study we investigated the effect of hyperhomocysteinemia on glutamate uptake in slices of parietal cortex of rats. First, we evaluated the effect of a single Hcy injection in 6-day-old rats, which possess a brain development similar to a human neonate (Clancy et al., 2007), and 29-day-old rats, evaluating an important period of cerebral development after birth. We observed a strong inhibition (approximately 59% and 40%, respectively) on glutamate uptake in slices of parietal cortex from 6- and 29-day-old rats subjected to a single Hcy injection. However, neonate rats did not recover the normal level of glutamate uptake until 30 days after Hcy injection; the oldest rats completely retrieved the glutamate uptake measured 12 h after a single administration of Hcy. These data suggest that when the brain is in development (6-day-old rats), a single Hcy administration is able to alter for a long term the system

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of glutamate uptake, while when the brain development was almost completed (29-day-old rats) the same stimulus by Hcy elicited a temporary and reversible neurotoxic effect.

Regarding chronic treatment, results showed that Hcy also reduced significantly the glutamate uptake in slices of parietal cortex from rats. This effect appears to be permanent, as far as until 30 days after the last injection of Hcy, we still observed the same level of inhibition on glutamate uptake found 12 h, 1 and 7 days after the last Hcy administration. Since acute studies showed that neonate rats were more vulnerable to Hcy effect, we could suggest that the effect observed in chronic hyperhomocysteinemia might be a result from the treatment beginning, when the rats are 6-day-old and the CNS development was uncompleted.

In order to investigate a possible mechanism for the reduction on glutamate uptake caused by chronic Hcy administration, we evaluated the immunocontent of GLAST and GLT-1, which account by the clearance of glutamate from the synaptic cleft and extrasynaptic compartment (Rothstein et al., 1994; Danbolt, 2001; Sheldon and Robinson, 2007). These high-affinity sodium-dependent glutamate transporters are present in high concentration in brain of humans and rats. GLAST could be found intracellularly in neurons, but mainly in glial cells over the brain, with a high prevalence in cerebellum; while GLT-1 is predominantly expressed in glial cells throughout the brain, as shown by immunocytochemical and histochemical methods, being responsible for up to 90% of all glutamate transport in adult tissue (Gegelashvili and Schousboe, 1998; Mennerick et al., 1998; Maragakis and Rothstein, 2004). GLAST and GLT-1 possess different pattern of expression, as showed by immunocytochemical localization studies performed in brain of rats during their development. GLAST is early expressed in forebrain when compared to GLT-1. GLAST already appear at birth, reaching comparable adult levels on their 30th day-of-life (Furuta et al., 1997; Ullensvang et al., 1997). In contrast, GLT-1 appears only after the first postnatal week, when its levels rise rapidly until around 30-day-old, when reach adult levels (Furuta et al., 1997; Ullensvang et al., 1997). Our results showed a substantial reduction in GLAST and GLT-1 immunocontent in parietal cortex of chronic hyperhomocysteinemic rats. We believe that this decrease in glutamate transporters is, at least in part, responsible by the great inhibition in glutamate uptake verified in our study. The down regulation of GLAST and GLT-1 could lead to increased extracellular glutamate levels (Maragakis and Rothstein, 2004; Sheldon and Robinson, 2007), contributing to excitotoxicity and neurodegeneration reported as Hcy effects (Kruman et al., 2000; Ho et al., 2002; Mattson et al., 2002).

Although some reports show that GLAST and GLT-1 expression could be positively or negatively regulated by pathways involving nuclear factor-kappaB (NFκB), Akt, extracellular signal-regulated kinases (Erk), and tumor necrosis factor-α (TNF-α) (Su et al., 2003; Dallas et al., 2007); the mechanism responsible by the reduction in glutamate transporters immunocontent elicited by Hcy administration has not been established yet. There are evidences suggesting that glutamate transporters could be inhibited by products of lipid peroxidation and free radicals, by direct oxidation of the transporter protein SH-groups (Volterra et al., 1994; Gegelashvili and Schousboe, 1997; Maragakis and Rothstein, 2004; Sheldon and Robinson, 2007). In this context, we recently showed that Hcy chronic treatment induced a consistent oxidative stress in parietal cortex of rats, increasing lipid peroxidation and DNA damage, and reducing enzymatic and non-enzymatic antioxidant defenses (Matté et al., 2007, 2009). In accord with our results, it has been reported the production of reactive species as a consequence of glutamatergic receptors activation by Hcy (Ho et al., 2003; Jara-Prado et al., 2003; Zieminska and Lazarewicz, 2006), as well as by Hcy autoxidation releasing superoxide and hydrogen peroxide (Ho et al., 2002; Dayal et al., 2004; Faraci and Lentz, 2004).

We suggest that the excessive stimulation of glutamatergic system, evoked by Hcy, could be a result of impaired glutamate clearance from the synaptic cleft caused by the inhibition of glutamate transporters, verified in our study. In this context, the overstimulation of glutamate receptors (Nicholls, 2008) and/or Hcy administration (Ho et al., 2002; Matté et al., 2007, 2009) induce free radical generation, which could be responsible by the inhibition of GLAST and GLT-1 transporters, impairing the glutamate clearance, and creating a circuit of excitotoxicity (Volterra et al., 1994; Maragakis and Rothstein, 2004; Sheldon and Robinson, 2007). However, we could not discard the direct effect of Hcy on glutamatergic receptors, promoting excitotoxicity by action as an agonist of NMDA receptor (Lipton et al., 1997; Jara-Prado et al., 2003; Zieminska et al., 2003; Zieminska and Lazarewicz, 2006; Poddar and Paul, 2009).

Another possible mechanism for glutamate uptake dysfunction is the inhibition of Na^+, K^+-ATPase activity, resulting in loss of the Na^+ concentration gradient. In this situation, GLAST and GLT-1, which are dependent of sodium-gradient, could reverse the direction of glutamate transport, resulting in accumulation of extracellular glutamate (Danbolt, 2001; Maragakis and Rothstein, 2004; Sheldon and Robinson, 2007; Nicholls, 2008). Consistent with these findings, we previous showed that Hcy chronic and acute administrations inhibited Na^+, K^+-ATPase activity in plasmatic membranes prepared from parietal cortex of rats (Matté et al., 2004, 2006, 2007), as well as in hippocampus (Streck et al., 2002; Wyse et al., 2002). Contributing with this hypothesis, a similar effect has been reported for ischemia (Rossi et al., 2000; Camacho and Massieu, 2006; Sheldon and Robinson, 2007), and some neurodegenerative diseases (Greenamyre et al., 1999; Higgins et al., 1999), that have been related to hyperhomocysteinemia (Mattson et al., 2002; Obeid et al., 2007).

To our knowledge, it was the first demonstration that Hcy inhibits glutamate uptake and reduces GLAST and GLT-1 immunocontent in parietal cortex of rats. Our results also bring a significant evidence indicating that immature CNS are more sensitive to Hcy than an older, showing the importance of starting the treatment for hyperhomocysteinemic patients as soon as possible, in order to mitigate the neurotoxic effects of Hcy.

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Capítulo V

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Concurrent folate treatment prevents Na^+,K^+-ATPase activity inhibition and memory impairments caused by chronic hyperhomocysteinemia during rat development

Cristiane Matté, Emilene B.S. Scherer, Francieli M. Stefanello, Alethéa G. Barschak, Carmen R. Vargas, Carlos A. Netto, Angela T.S. Wyse *

Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil

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Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil

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Abstract

We investigated the hypothesis that folate administration would prevent hyperhomocysteinemia-induced memory deficits and Na^+,K^+-ATPase activity inhibition. Chronic hyperhomocysteinemia was induced from the 6th to the 28th day of life by subcutaneous injection of homocysteine (0.3–0.6 μmol/g), twice a day; control Wistar rats received the same volume of saline solution (0.9% NaCl). Half of the homocysteine- and saline-treated groups also received intraperitoneal administration of folate (0.011 μmol/g) from the 6th to the 28th day of life. A group of animals was killed 12 h after the last injection, plasma and parietal cortex were collected for biochemical analysis. Another group stayed at Central Animal House until 60th day of life, when the rats were submitted to behavioral testing in water maze or were killed for evaluation of cortical Na^+,K^+-ATPase activity. Results showed that hyperhomocysteinemia impaired reference memory for platform location, as assessed by fewer crossings to the platform place and increased latency for the first crossing, when compared to controls. In the working memory task homocysteine-treated animals also needed more time to find the platform. We also observed that Na^+,K^+-ATPase activity was reduced in parietal cortex of hyperhomocysteinemic rats sacrificed 12 h after the last injection of homocysteine (29-day-old rats). In contrast, this enzyme was not altered when the rats were sacrificed 31 days after the treatment (60-day-old rats). Hyperhomocysteinemic rats treated with folate had all those impairments prevented, an effect probably related to folate antioxidant properties.

Keywords: Homocysteine; Folate; Memory and cognitive deficits; Na^+,K^+-ATPase; Parietal cortex

1. Introduction

Hyperhomocysteinemia occurs in various pathologies involving the central nervous system such as stroke, dementia and Alzheimer's disease (Clarke et al., 1998; Yoo et al., 1998; Eikelboom et al., 2000; Loscalzo, 2002; Seshadri et al., 2002; Mattson et al., 2002; Morris, 2003; Dwyer et al., 2004; Troen, 2005; Obeid and Herrmann, 2006). Tissue accumulation of homocysteine (Hcy) also occurs in homocystinuria, a metabolic disease caused by severe cystathionine-β-synthase deficiency. Affected patients present mental retardation, seizures and are more susceptible to heart disease and stroke (Mudd et al., 2001). Although neurological dysfunction is found in a considerable number of patients, the exact mechanism(s) involved remains poorly understood.

Since the strategy of using animal models is useful to better understand the pathophysiology of diseases, we have developed a chemical experimental rat model of hyperhomocysteinemia (Streck et al., 2002) in which the plasma Hcy concentration is increased to levels that are similar to those found in human homocystinuria (Mudd et al., 2001). Animals subjected to this experimental model present impairment of aversive and spatial memory (Reis et al., 2002; Streck et al., 2004).

Na^+,K^+-ATPase is responsible for generating and maintaining membrane potential necessary for neuronal excitability and thus disturbances in its activity could have grave consequences

Abbreviations: Hcy, homocysteine; CNS, central nervous system; LTP, long-term potentiation; TBARS, thiobarbituric acid-reactive substances; LC–MS/MS, liquid chromatography electrospray tandem mass spectrometry; Pi, inorganic phosphate; MDA, malondialdehyde; ANOVA, analysis of variance; SPSS, Statistical Package for the Social Sciences.

* Corresponding author. Tel.: +55 51 3308 5573; fax: +55 51 3308 5535.
E-mail address: wyse@ufrgs.br (A.T.S. Wyse).

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for central nervous system (CNS) functioning (Ericinska and Silver, 1994; Mobasheri et al., 2000). It has been reported that Na^+,K^+-ATPase might play a relevant role in mechanism for learning (Brunelli et al., 1997), as well as in activity-dependent synaptic plasticity, such as long-term potentiation (LTP) (Glushchenko and Izvarina, 1997). In this context, Na^+,K^+-ATPase inhibition by ouabain impairs learning and memory in Morris water maze (Zhan et al., 2004) and step-through passive avoidance tasks (Gibbs and Ng, 1977; Ng and Gibbs, 1991; Sato et al., 2004), showing the main role of this enzyme on learning and memory. Wyse et al. (2004) also showed that Na^+,K^+-ATPase activity may be involved in the memory consolidation of step-down inhibitory avoidance in the hippocampus.

Folate is a cofactor of the enzyme reaction that promotes remethylation of Hcy (Fowler, 1997; Finkelstein, 1998; Brosnan et al., 2004). This vitamin is also essential to DNA synthesis and repair, as well as to amino acid synthesis (Mattson and Haberman, 2003; Brosnan et al., 2004). Folate deficiency results in DNA strand breaks (Pogribny et al., 1995; Fenech, 2001), DNA hypomethylation (Pogribny et al., 1997; Fenech, 2001) and abnormal gene expression (Wainfan and Poirier, 1992). Furthermore, emerging evidence relates elevated levels of Hcy and/or low serum folate levels with higher rates of vascular, neurodegenerative and neuropsychiatric diseases, as well as with neural tube defects, Down's syndrome, cancer and homocystinuria (Brattstrom et al., 1988; Boushey et al., 1995; Daly et al., 1995; Rydlewicz et al., 2002; Mattson et al., 2002; Mattson, 2003; Fillon-Emery et al., 2004; Lamers et al., 2004; Bottiglieri, 2005; Verhoef and de Groot, 2005). On the other hand, it has been shown that folate supplementation can significantly reduce the risk of these disorders (Botez et al., 1982; Daly et al., 1995; Kim, 1999; Mattson et al., 2002; Smith and Bodamer, 2002; Verhaar et al., 2002; Mattson and Shea, 2003; Bottiglieri, 2005; Moore, 2005; Eskes, 2006). In this context, our laboratory has recently reported that folate pretreatment prevents the inhibition of Na^+,K^+-ATPase activity caused by acute hyperhomocysteinemia in rats (Matté et al., 2006).

In the present work we investigated whether folate treatment would affect cognitive performance of rats subjected to chronic hyperhomocysteinemia measured on spatial reference and working memory tasks. We also evaluated the influence of folate on Na^+,K^+-ATPase activity inhibition caused by chronic Hcy administration. The hypothesis is that folate treatment would prevent the impairments caused by hyperhomocysteinemia.

2. Methods and materials

2.1. Animals and reagents

Male Wistar rats were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental and was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil.

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Homocysteine and folate administration

Hcy and folate were dissolved in 0.9% NaCl and the pH adjusted to 7.4 with 0.1 M NaOH. Hcy solution was administered subcutaneously twice a day at 8 h intervals from 6th to 28th day of age. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Streck et al., 2002). During the first week of treatment, animals received 0.3 μmol Hcy/g body weight. In the second week, 0.4 μmol Hcy/g body weight was administered to the animals, and in the last week rats received 0.6 μmol Hcy/g body weight. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those found in homocystinuric patients. Folate solution (0.011 μmol folate/g body weight) was injected intraperitoneally, once a day, from 6th to 28th day of life (adapted from Lalonde et al., 1993). Control animals received saline solution (0.9% NaCl) in the same volumes as those applied to Hcy and folate-treated rats.

A group of rats was sacrificed by decapitation without anesthesia 12 h after the last injection of Hcy and/or folate. Parietal cortex and plasma were collected for posterior biochemical parameters determination. Another group of animals stayed at Central Animal House of the Biochemistry Department until 60th day of life, when they were submitted to behavioral testing or were sacrificed, parietal cortex was dissected to synaptic plasma membranes preparation to Na^+,K^+-ATPase determination.

2.3. Homocysteine levels determination

Hcy levels in plasma and parietal cortex were determined as described by Magera et al. (1999), 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 internal standard.

2.4. Behavioral procedures

On the 60th day of life, animals were subjected to behavioral testing. We used the Morris water maze, an apparatus widely employed for the study of spatial learning and memory tasks that depend on hippocampal function (Morris et al., 1982; Netto et al., 1993; D'Hooge and De Deyn, 2001).

The water maze consisted of a black round tank, 200 cm in diameter and 100 cm high, filled to a depth of 50 cm with water, maintained at constant temperature of 23 °C. The tank was theoretically divided into four equal quadrants for the purpose of analysis. Several distal visual cues were placed on the walls of the room. Trials were recorded by a video camera mounted above the center of the tank.

(a) *Reference memory task.* The task consisted of five training and one test session. In the acquisition phase, rats had daily sessions of four trials per day for 5 days to find the platform, submerged 2 cm under the water surface, placed on the center of one of the quadrants of the tank during all training days. For each trial, the rat was placed in water facing tank wall, in one of the four starting locations (N, S, W and E). The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and, in the case of failing to find it, they were gently guided to it; all animals were permitted to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was 15–20 min. One day after the last training trial, each rat was subjected to a probe trial in which the platform was removed. We measured four parameters, namely latency to cross on the location of the platform, the number of target crossings and the time spent in target (the quadrant in which the platform was located in the training sessions)

and opposite quadrants. These parameters were taken as a measure for spatial memory (Netto et al., 1993).

In order to detect motor impairments that could affect performance in experimental groups, the swimming speed was calculated by taking the distance traveled in the first 15 s of the probe trial.

(b) *Working memory task.* After 1 week, the working memory version of Morris water maze was performed. The task consisted of four consecutive trials per day, with a 30-s inter-trial interval, when the animals were placed in the tank facing the wall and allowed to search for the submerged platform, positioned on the center of one of the quadrants. Platform position changed every subsequent day during the four testing days. Latencies to find the platform in every first, second, third and fourth trials were calculated considering all testing days so to assess working memory performance (Netto et al., 1993).

(c) *Open field task.* The task was run in a wooden box measuring 60 cm × 40 cm × 50 cm with a frontal glass wall, whose floor was divided by white lines into 12 equal squares. Animals were placed facing the rear left corner of the arena and observed for 2 min. The number of squares crossed with the four paws from one square to another was indicative of motor activity (Netto et al., 1986).

2.5. Preparation of synaptic plasma membranes from parietal cortex

For preparation of synaptic plasma membranes and determination of Na^+,K^+-ATPase activity, the parietal cortex of rats was homogenized in 10 volumes (1:10, w/v) of 0.32 M sucrose solution containing 5.0 mM HEPES and 0.1 mM EDTA, pH 7.4. Synaptic plasma membrane from parietal cortex was prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al., 1998). The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant removed and centrifuged at 12,000 × *g* for 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris–HCl buffer, pH 8.1) at 0 °C for 30 min, and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000 × *g* for 120 min, the fraction between 0.8 and 1.0 M sucrose interface was taken as the membrane enzyme preparation.

2.6. Na^+,K^+-ATPase activity assay

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 final volume of 200 μL. The reaction was initiated by ATP addition. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays, as described by Wyse et al. (1998). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific enzyme activity was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicate.

2.7. Thiobarbituric acid-reactive substances assay

TBARS, an index of lipid peroxidation, was determined according to the method described by Ohkawa et al. (1979). Briefly, parietal cortex was homogenized in 10 volumes (1:10, w/v) of 1.15% KCl, and centrifuged at 1000 × *g* for 10 min. Fifty microliters of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid were added to 500 μL of supernatant in a Pyrex tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifuged at 1000 × *g* for 10 min. The organic layer was taken and the resulting pink color was determined in a spectrophotometer at 535 nm. The results are reported as nmol of malondialdehyde (MDA) per mg of protein. All samples were run in triplicate.

2.8. Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) or Bradford (1976) using bovine serum albumin as standard.

2.9. Statistical analysis

Reference memory training and working memory data were analyzed by repeated measure analysis of variance (ANOVA) and data from the probe trial parameters, open field test, Hcy levels, TBARS, and Na^+,K^+-ATPase activity data were analyzed by Student's *t* test or by one-way ANOVA; *post hoc* Duncan's multiple range test was run when indicated. Descriptive statistics data were expressed as mean ± S.E.M., as indicated. Type error rate was set at 0.05 for determining statistical significance, where multiple range test was used for *post hoc* testing. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, in a PC-compatible computer.

3. Results

3.1. Effect of chronic administration of homocysteine, folate, and homocysteine plus folate on reference and working memory tasks in the Morris water maze

Fig. 1 shows that Hcy did not affect learning of platform spatial position in the acquisition phase, when compared to control group performance. Likewise, the performance of folate- and Hcy plus folate-treated rats did not differ from control animals along this phase (day 1 [$F(3,51) = 0.72$; $p > 0.05$], day 2 [$F(3,51) = 1.06$; $p > 0.05$], day 3 [$F(3,51) = 1.01$; $p > 0.05$], day 4 [$F(3,51) = 0.56$; $p > 0.05$], and day 5 [$F(3,51) = 1.63$; $p > 0.05$]).

The probe trial was run 24 h after acquisition phase, when four parameters were evaluated: the number of crossings over and the latency to cross the platform location, and the time spent in target and opposite quadrants (Fig. 2A–D). Its showed that Hcy administration significantly reduced the number of crossings over (Fig. 2A) [$F(3,51) = 6.68$; $p < 0.01$], and increased the latency to cross (Fig. 2B) [$F(3,51) = 5.58$; $p < 0.01$] the platform location. However, folate treatment prevented the increase in the latency to cross the platform place caused by Hcy administration (Fig. 2B). In addition, Hcy treatment did not alter other parameters evaluated in the probe trial: time spent in the target quadrant (C) [$F(3,51) = 1.37$;

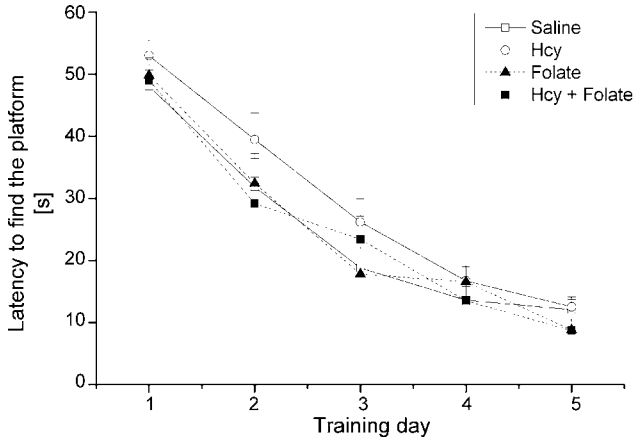

Fig. 1. Effect of chronic homocysteine, folate, and homocysteine plus folate administration on performance of spatial memory acquisition phase. Data shows latency to find the platform across blocks of four trials on each day and are expressed as mean ± S.E.M. for 12–15 animals in each group. There were no significant differences between groups (repeated measures ANOVA). Hcy: homocysteine.

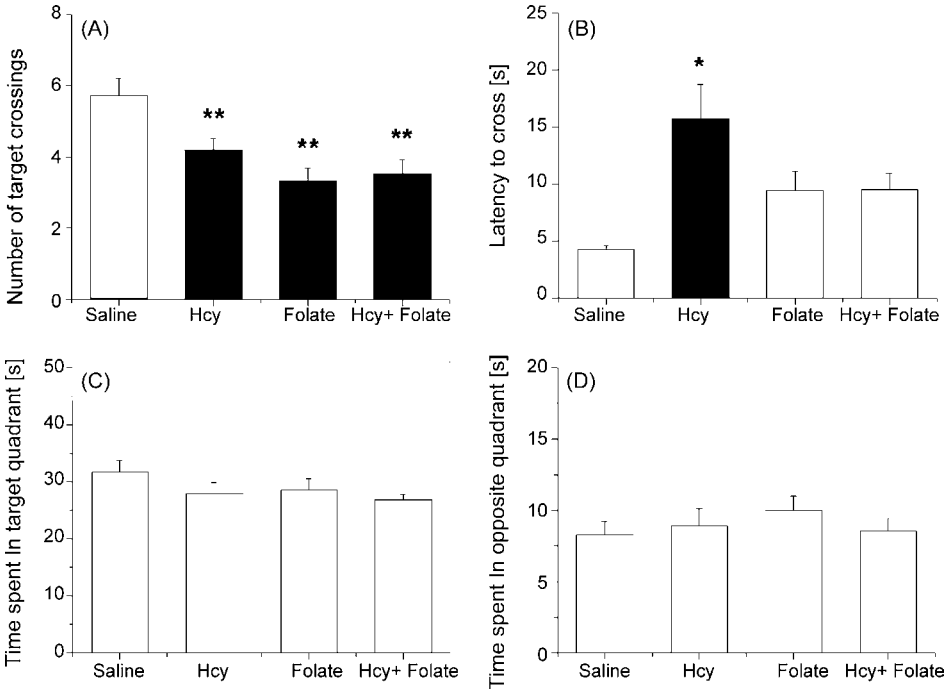

Fig. 2. Effect of chronic homocysteine, folate, and homocysteine plus folate administration on test session parameters, namely: number of target crossings over platform location (A), latency to cross the location of the platform (B), time spent in the target quadrant (C), and time spent in the opposite quadrant (D). Data are expressed as mean ± S.E.M. for 12–15 animals on each group. Different from control, $*p < 0.05$ and $**p < 0.01$ (repeated measures ANOVA). Hcy: homocysteine.

$p > 0.05$], and in the opposite quadrant (D) [$F(3,51) = 0.46$; $p > 0.05$].

It was also shown that chronic Hcy administration affected working memory in the Morris water maze (Fig. 3). Hcy effect was major on trials 1 [$F(3,51) = 2.66$; $p < 0.05$] and 3 [$F(3,51) = 2.68$; $p < 0.05$]; nevertheless its effect was prevented by concurrent folate administration. ANOVA showed that Hcy did not alter performance of rats on trials 2 [$F(3,51) = 1.02$; $p > 0.05$] and 4 [$F(3,51) = 0.60$; $p > 0.05$] of working memory.

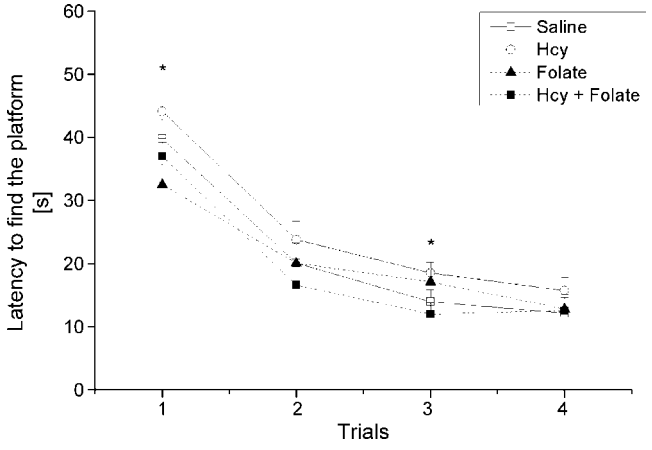

Fig. 3. Effect of chronic homocysteine, folate, and homocysteine plus folate administration on performance on working memory version of Morris water maze. Data are latencies to find the platform on each trial during the 4 days and are expressed as mean ± S.E.M. for 12–15 animals in each group. Different from control, $*p < 0.05$ (repeated measures ANOVA). Hcy: homocysteine.

Table 1 shows no motor deficits in any experimental group as evaluated by ANOVA [$F(3,8) = 0.286$; $p > 0.05$] of swim speed data.

Rats chronically treated with Hcy did not significantly differ from saline-, folate-, and Hcy plus folate-treated rats with respect to body weight, both immediately after the end of treatment (28-day-old age) and before behavioral tests (60-day-old age), indicating no evidence of malnutrition (data not shown).

3.2. Effect of chronic administration of homocysteine, folate, and homocysteine plus folate on open field task

In order to further confirm the absence of motor effects caused by distinct treatments, we submitted all groups (saline, Hcy, folate, and Hcy plus folate) to the open field task. Fig. 4 shows that all groups had similar performance in the number of crossings [$F(3,11) = 0.159$; $p > 0.05$].

Table 1
Effect of chronic administration of homocysteine, folate and homocysteine plus folate on swim speed of rats in Morris water maze task

Group	Swim speed in Morris water maze task (cm/s)
Saline	35.57 ± 1.0
Homocysteine	34.05 ± 1.2
Folate	35.37 ± 2.5
Homocysteine + folate	33.94 ± 1.3

Data are expressed as mean ± S.E.M. for three rats in each group. There was no significant difference between groups (Duncan's multiple range test).

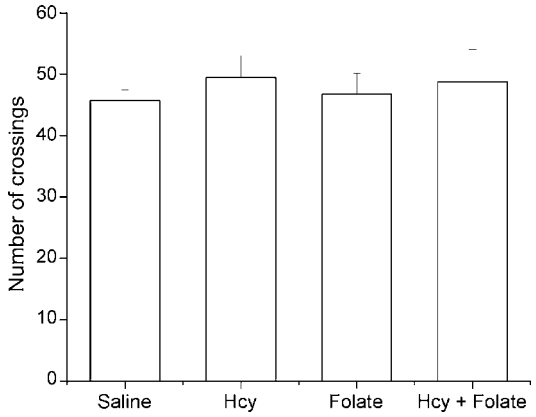

Fig. 4. Effect of chronic homocysteine, folate, and homocysteine plus folate administration on performance on open field task. Data are the number of line crossings during 120 s and are expressed as mean ± S.E.M. for 13–15 animals in each group. There were no significant differences between groups (one-way ANOVA). Hcy: homocysteine.

3.3. Effect of chronic administration of homocysteine, folate, and homocysteine plus folate on Na^+,K^+-ATPase activity in parietal cortex of rats

Results show that hyperhomocysteinemic rats sacrificed 12 h after the last injection of Hcy presented a significant reduction of Na^+,K^+-ATPase activity in synaptic plasma membranes from parietal cortex (Fig. 5A). *Post hoc* analysis showed that treatment with folate *per se* did not alter this enzyme activity, but prevented the reduction of Na^+,K^+-ATPase activity caused by chronic hyperhomocysteinemia [$F(3,12) = 4.482$; $p < 0.05$]. On the other hand, there was no alterations of Na^+,K^+-ATPase activity in parietal cortex of rats subjected to chronic hyperhomocysteinemia sacrificed 31 days after the last administration of Hcy [$t(6) = 0.186$; $p > 0.05$] (Fig. 5B).

3.4. Effect of folate treatment on Hcy levels in plasma and parietal cortex of rats subjected to hyperhomocysteinemia

Table 2 shows that Hcy administration increased significantly the levels of Hcy in plasma [$F(3,14) = 150.37$; $p < 0.001$] and parietal cortex [$F(3,12) = 8.55$; $p < 0.01$] of rats subjected to chronic hyperhomocysteinemia. *Post hoc* analysis showed that folate concurrent treatment did not reduce Hcy levels, when compared to Hcy-treated rats.

3.5. Effect of folate chronic administration on thiobarbituric acid-reactive substances in parietal cortex of hyperhomocysteinemic rats

Considering that folate did not alter plasma and cerebral Hcy levels in hyperhomocysteinemic rats and that this vitamin has antioxidant properties, we also tested the effect of Hcy and folate administration on TBARS, an index of lipid peroxidation, in parietal cortex of rats. Table 3 shows that Hcy significantly increased and folate reduced TBARS (28%) as compared to control; folate when administered concurrent with

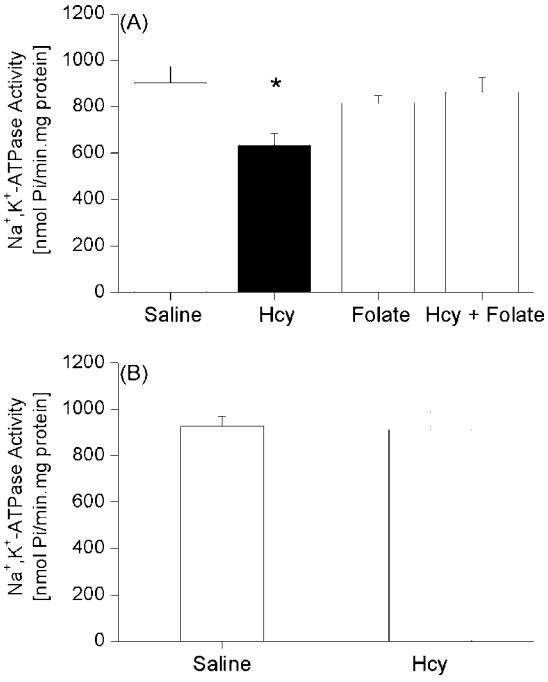

Fig. 5. Effect of homocysteine and/or folate administration on Na^+,K^+-ATPase activity in synaptic plasma membrane from parietal cortex of rats with 29 days-old (A) and 60 days-old (B) subjected to chronic hyperhomocysteinemia from 6th to 28th day of age. Results are mean ± S.E.M. for four animals in each group. Different from control, $*p < 0.05$ Student's t or ANOVA, (Duncan's multiple range test). Hcy: homocysteine.

Hcy prevented the increase of TBARS caused by hyperhomocysteinemia [$F(3,16) = 13.78$; $p < 0.001$].

4. Discussion

We investigated the action of folate on the effects elicited by chronic Hcy administration on rat cognitive performance assessed with the Morris water maze.

Results showed that hyperhomocysteinemia impaired reference memory of the platform location and that Hcy-treated animals presented fewer crossings to the place where the platform was located in training trials and increased the latency to cross the platform place when compared to saline-treated animals (controls). Results also showed that Hcy-treated animals demonstrated a reduced efficiency to find the platform

Table 2
Effect of chronic administration of homocysteine, folate and homocysteine plus folate on homocysteine levels in rats

Group	Plasma homocysteine levels (μM)	Parietal cortex homocysteine levels (μM)
Saline	<2.5 ± 0.0	10.5 ± 1.0
Homocysteine	123 ± 5.2**	16.0 ± 0.8*
Folate	<2.5 ± 0.0	10.4 ± 1.5
Homocysteine + folate	140 ± 12.2**	17.9 ± 1.7*

Data are expressed as mean ± S.E.M. for 4–5 rats in each group. Different from control (saline) and folate, $*p < 0.01$ and $**p < 0.001$ (Duncan's multiple range test).

Table 3
Effect of folate chronic administration on thiobarbituric acid-reactive substances (TBARS) in parietal cortex of hyperhomocysteinemic rats

Group	TBARS (nmol MDA/mg protein)
Saline	4.04 ± 0.31 a
Homocysteine	4.84 ± 0.18 b
Folate	2.91 ± 0.24 c
Homocysteine + folate	3.26 ± 0.16 c

Data are expressed as mean ± S.E.M. for five rats in each group. $p < 0.001$ (Duncan's multiple range test).

position in the working memory task, suggesting a deficit in spatial navigation (D'Hooge and De Deyn, 2001). Hcy-induced deficits cannot be attributed to a decrease in locomotor activity since the swim speed (general mean of 34.73 cm/s) of all groups did not differ and there were no difference between groups in the number of line crossings in the open field task. These results confirmed our previous studies, showing that Hcy cause an impairment spatial memory in rat (Streck et al., 2004).

Since Na^+,K^+-ATPase is inhibited by chronic hyperhomocysteinemia in rat brain (Streck et al., 2002; Matté et al., 2004) and there are evidences showing that this enzyme might play a relevant role in mechanism for learning (Gibbs and Ng, 1977; Ng and Gibbs, 1991; Brunelli et al., 1997; Sato et al., 2004; Wyse et al., 2004; Zhan et al., 2004), we also investigated the Na^+,K^+-ATPase activity in parietal cortex of chronic hyperhomocysteinemic rats at different post-last injection periods. Results showed that chronic hyperhomocysteinemia reduces Na^+,K^+-ATPase activity when the rats were sacrificed 12 h after the last injection of Hcy, but not when the animals were sacrificed 31 days after the treatment. These results indicate that the hyperhomocysteinemia inhibited Na^+,K^+-ATPase and that 31 days after Hcy treatment Na^+,K^+-ATPase activity returned to normal, what could suggest long-term modulation of its activity (Bertorello and Kats, 1995). Along this reasoning, this effect could be attributed to: (a) enzyme degradation during the Hcy treatment, when its synthesis is turned off; (b) microenvironmental changes caused by degradation of membrane phospholipids surrounding enzyme molecule due to lipid peroxidation, as demonstrated by Hcy *in vitro* (Streck et al., 2003; Matté et al., 2004).

Recent clinical evidence has shown that folate deficiency increases the risk of stroke, as well as Parkinson's and Alzheimer's diseases (Mattson et al., 2002) and that the supplementation of this vitamin can significantly attenuate the clinical features of homocystinuria and of various neurodegenerative disorders (Kim, 1999; Mattson et al., 2002; Verhaar et al., 2002; Mattson and Shea, 2003; Bottiglieri, 2005; Moore, 2005). In this context, some reports have shown that folic acid administration can reduce blood Hcy concentrations both in humans and rats (Homocysteine Lowering Trialists' Collaboration, 1998; Rydlewicz et al., 2002; Lamers et al., 2004). In addition, there is strong evidence indicating a potent antioxidant effect of this vitamin, which is independent of its effect on Hcy metabolism (Doshi et al., 2001; Nakano et al., 2001). Therefore, since in our study folate treatment did not decrease Hcy concentration in plasma and parietal cortex, we can discard the possibility that folate pretreatment provided the cells with sufficient pool of methyl-tetrahydrofolate, a coenzyme critical in conversion of Hcy to methionine. These results are in agreement with evidences showing that folate could act through still unknown mechanisms like it does in other forms of neurodegeneration (Mattson et al., 2002; Mattson and Shea, 2003; Bottiglieri, 2005) and by inhibiting the decrease of cognition in aging (Kado et al., 2005).

There is good evidence that oxidative stress and reactive oxygen species might be involved in inhibition of Na^+,K^+-ATPase and in memory modulation mechanisms (Dobrota et al., 1999; Kurella et al., 1999; Bickford et al., 2000; Cantuti-Castelvetri et al., 2000; Wang et al., 2003; Chakraborty et al., 2003; Silva et al., 2004; Abidin et al., 2004; Butterfiel et al., 2006; Foster, 2006). Another line of evidence supporting the role of oxidative stress in behavior emerges from studies with vitamins (Chan et al., 2004; Monteiro et al., 2005; Delwing et al., 2006). For example, α-tocopherol improves cognitive function of patients with temporal lobe radionecrosis (Chan et al., 2004) and may be beneficial in lowering the cognitive impairment in patients with Alzheimer's disease (Mecocci et al., 2004). In this context, previous results have demonstrated that Hcy induces *in vitro* brain oxidative stress (Wyse et al., 2002; Streck et al., 2003; Matté et al., 2004) and that vitamins E and C are able to prevent the impairment of memory caused by acute hyperhomocysteinemia, suggesting the participation of oxidative stress in such effects (Reis et al., 2002). In addition, there is also strong evidence indicating a potent antioxidant effect of folate, which is independent of its effect on Hcy metabolism (Doshi et al., 2001; Nakano et al., 2001).

Since we have previously reported that Hcy increases lipid peroxidation (Matté et al., 2004) and in order to understand the mechanisms by which folate was effective in preventing memory deficits and the inhibition of Na^+,K^+-ATPase activity, we also studied the effect of this vitamin on TBARS, an index of lipid peroxidation, in parietal cortex of hyperhomocysteinemic rats. Results showed that folate significantly prevented the increase in TBARS levels found in parietal cortex of hyperhomocysteinemic rats, suggesting that oxidative stress is, at least in part, associated with neurochemical (Na^+,K^+-ATPase) and behavioral (spatial memory) changes observed in our study.

In conclusion, our results showed that folate prevented the reduction of Na^+,K^+-ATPase activity and spatial memory impairment caused by chronic hyperhomocysteinemia in rats, an effect probably related to folate antioxidant properties. Further studies are necessary to clarify the mechanisms underlying such prevention and its relevance as adjuvant therapy to specific diets in hyperhomocysteinemic patients.

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Capítulo VI

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ACUTE HOMOCYSTEINE ADMINISTRATION IMPAIRS MEMORY CONSOLIDATION ON INHIBITORY AVOIDANCE TASK AND DECREASES HIPPOCAMPAL BRAIN-DERIVED NEUROTROPHIC FACTOR IMMUNOCONTENT: PREVENTION BY FOLIC ACID TREATMENT

C. MATTÉ, L. O. PEREIRA, T. M. DOS SANTOS, V. MACKEDANZ, A. A. CUNHA, C. A. NETTO AND A. T. S. WYSE*

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

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

Abstract—In the present study, we first investigated the effect of single homocysteine administration on consolidation of short- and long-term memories of inhibitory avoidance task in Wistar rats. We also measured brain-derived neurotrophic factor levels in the hippocampus and parietal cortex of rats. The influence of pretreatment with folic acid on behavioral and biochemical effects elicited by homocysteine was also studied. Wistar rats were subjected to a folic acid or saline pretreatment from their 22nd to 28th day of life; 12 h later they were submitted to a single administration of homocysteine or saline. For motor activity and memory evaluation we performed open-field and inhibitory avoidance tasks. Hippocampus and parietal cortex were obtained for brain-derived neurotrophic factor immunocontent determination. Results showed that homocysteine impaired short- and long-term memories and reduced brain-derived neurotrophic factor levels in the hippocampus. Pretreatment with folic acid prevented both the memory deficit and the reduction in the brain-derived neurotrophic factor immunocontent induced by homocysteine injection. Further studies are required to determine the entire mechanism by which folic acid acts and its potential therapeutic use for memory impairment prevention in homocystinuric patients.

Key words: homocysteine, folic acid, memory, BDNF, parietal cortex, hippocampus.

Homocystinuria, an inborn error of metabolism caused by cystathionine β-synthase deficiency, is biochemically characterized by high homocysteine (Hcy) plasma levels. Patients frequently present mental retardation, seizures, and vascular complications (Mudd et al., 2001). Moreover, hyperhomocysteinemia has emerged as an important risk factor for neurodegenerative and vascular diseases (Diaz-Arrastia, 2000; Mattson et al., 2002; Sachdev, 2004, 2005).

Memory is a dynamic process consisting of at least three phases: acquisition, consolidation, and retrieval (Izquierdo, 2002). There is evidence suggesting that memories are stored in brain synapses and that synaptic plasticity is thought to be the cellular mechanism for learning and memory (Lu et al., 2008). Considering the phenomenology, memory can be classified by its duration: short-term memory (STM), which lasts minutes to 1–3 h, and long-term memory (LTM), that lasts hours to days or years. They are not sequential stages of a single memory, but occur in parallel and by different molecular pathways. Furthermore, the first is independent of gene expression and protein synthesis, while the last is dependent on both mechanisms (Izquierdo et al., 2002).

Brain-derived neurotrophic factor (BDNF) is a neurotrophin released by neurons in an activity-dependent manner and is largely implicated in consolidation process in STM and LTM (Alonso et al., 2002, 2005; Bramham and Messaoudi, 2005; Soulé et al., 2006; Bekinschtein et al., 2008). BDNF secretion is regulated by neuronal activity, which means that it acts locally and specifically at active synapses, modulating synaptic activity and neural connectivity (Thomas and Davies, 2005; Bekinschtein et al., 2008). BDNF and tropomyosin-related kinase B receptor (TrkB) are colocalized both presynaptically and postsynaptically in glutamatergic synapses in many structures of the brain, including hippocampus and cerebral cortex (Carvalho et al., 2008; Bekinschtein et al., 2008). TrkB activation by BDNF triggers the activation of one or more of the three signaling pathways mediated by phosphatidyl-inositol 3 kinase, phospholipase Cγ and extracellular signal-regulated kinase 1/2. Some important actions of BDNF-TrkB in memory consolidation include glutamate release facilitation, phosphorylation and trafficking modulation of *N*-methyl-D-aspartate and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors, cAMP response element binding (CREB) phosphorylation leading to regulation of transcription, and modulation of dendritic synthesis of plasticity-related proteins, including calcium/calmodulin-dependent protein kinase II and Arc (Bramham and Messaoudi, 2005; Soulé et al., 2006; Bekinschtein et al., 2008; Tyler et al., 2008).

Hcy impairs memory in experimental models and has been related to cognitive dysfunction in humans (Reis et

*Corresponding author. Tel: +55-51-3308-5573; fax: +55-51-3308-5535.
E-mail address: wyse@ufrgs.br (A. T. S. Wyse).

Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element binding; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; Hcy, homocysteine; LTM, long-term memory; STM, short-term memory; TrkB, tropomyosin-related kinase B receptor.

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al., 2002; Streck et al., 2004; McCaddon, 2006; Troen et al., 2008). In this context, we developed a chemically induced model of hyperhomocysteinemia (Streck et al., 2002) and have been describing the effects of Hcy administration in memory of rats. Reis et al. (2002) showed that Hcy impairs LTM in inhibitory avoidance task; also, Streck et al. (2004) identified a spatial memory deficit in hyperhomocysteinemic rats submitted to Morris water maze. On the other hand, folic acid, a methyl donor in the Hcy remethylation pathway, appears to cause cognitive benefit in clinical and experimental studies (Kado et al., 2005; Durga et al., 2007; Matté et al., 2007). We have previously shown that this B-vitamin prevented Hcy-induced deficit on spatial learning, as measured in the Morris water maze task (Matté et al., 2007).

In the present study, we focused our attention on the effect of Hcy on memory consolidation. First, we evaluated the effect of Hcy acute administration on memory performance in inhibitory avoidance task, 1 (STM) or 24 h (LTM) after one immediate post-training Hcy-injection. We also investigated the effect of Hcy on BDNF levels in the hippocampus and parietal cortex of rats after a training session. The influence of pretreatment to the rats with folic acid on behavioral and biochemical effects elicited by Hcy administration was also studied.

EXPERIMENTAL PROCEDURES

Animals and reagents

Two hundred twenty-two male Wistar rats (22 days of age) were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. The animals were maintained on a 12-h light/dark cycle in an air-conditioned constant temperature (22±1 °C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996), and the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental were followed in all experiments. The study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil. All efforts were made to minimize the number of animals used and their suffering in our study.

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA; except the BDNF Emax ImmunoAssay System, which was purchased from Promega (Madison, WI, USA), and folate automated chemiluminescence system (ACS: 180), which was purchased from Siemens Medical solution Diagnostics (Puteaux, France).

Drug administration procedure

Animals were randomly assigned to one of the following groups: control (saline), folic acid, Hcy, and folic acid plus Hcy. Folic acid and D,L-Hcy were dissolved in 0.9% NaCl solution and buffered to pH 7.4. Animals were pretreated daily with an i.p. injection of folic acid (0.011 μmol/g body weight) or saline (0.9% NaCl) from their 22nd to their 28th day of life (Matté et al., 2006).

Twelve hours after the last injection (folic acid or saline), a group of rats received an s.c. administration of Hcy (0.6 μmol/g of weight body) or saline and was submitted to open-field task 1 h later (Fig. 1A). Hcy reaches the brain maximum concentration between 15 and 30 min after injection (Streck et al., 2002).

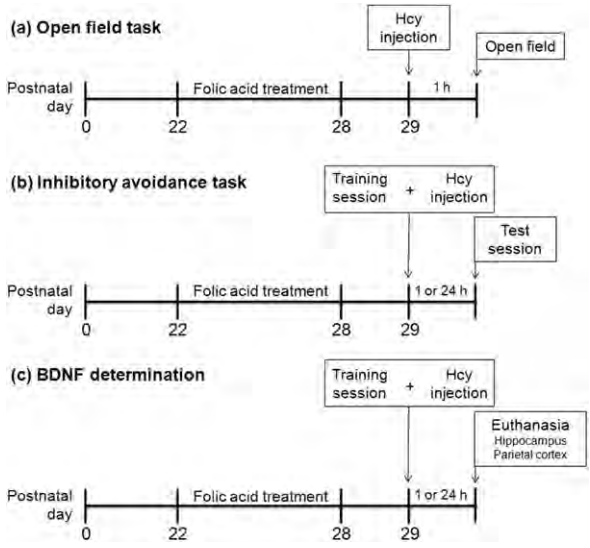

Fig. 1. Timeline of experimental procedures.

Another group of rats was submitted to a training session of inhibitory avoidance task, 12 h after folic acid or saline treatment. Immediately after the foot shock, rats received a single s.c. injection of Hcy (0.6 μmol/g of weight body) or saline (Streck et al., 2002) and were returned to their home cages until the test session of inhibitory avoidance task (Fig. 1B); or were euthanized 1 or 24 h after the Hcy injection, when the brain was quickly removed, and the hippocampus and parietal cortex were dissected and stored at −70 °C until BDNF measurement (Fig. 1C).

For folic acid determination, a group of rats (29 days old) was subjected to a single injection of folic acid (0.011 μmol/g body weight) and sacrificed 0 min, 15 min, 1 h, or 3 h later. Heparinized blood was collected and plasma was separated for folate measurement.

Open-field task

The task was run in a wooden box measuring 60×40×50 cm, with a frontal glass wall, and whose floor was divided by white lines into 12 equal squares. Animals were placed facing the rear left corner of the arena and observed for 3 min. The number of squares crossed with the four paws from one square to another was indicative of motor activity (Netto et al., 1986). After each trial, the apparatus was cleaned with an ethanol solution (20%). All animals were tested only once.

Step-down inhibitory avoidance task

Inhibitory avoidance task was performed in accordance with Izquierdo et al. (1997) and Wyse et al. (2004). The rats were gently placed on a 2.5-cm high, 7.0-cm wide, 25-cm long platform at the right side of a 50×25×25-cm apparatus, the floor of which contained a series of parallel 0.1-cm caliber stainless steel bars spaced 1.0 cm apart. In the training session (between 7:00 and 9:00 AM), after the rats stepped down placing the four paws on the grid, they received 3×0.4 s, 0.6 mA foot shock, immediately received a saline or Hcy injection, and were returned to their home cages. The test session was carried out 1 or 24 h after training session. The testing session was procedurally identical to the training session, except that no foot shock was given, and the step-down latency was measured (cutoff at 180 s). Previous to the experimental procedure, we performed a foot shock standard curve, between 0.2 and 0.7 mA (3×0.4 s). The young rats had a better performance in test session (1 or 24 h later) when trained with

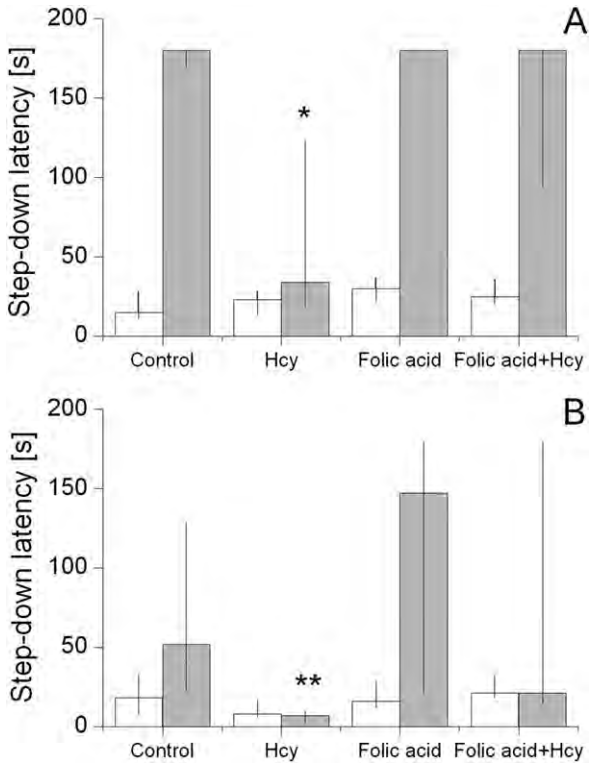

Fig. 2. Effect of folic acid pretreatment on performance of step-down inhibitory avoidance task of hyperhomocysteinemic rats tested 1 h (A) or 24 h (B) after the training session. White bars represent step-down latency on training session, while gray bars represent step-down latency on test session. Results are expressed as median (interquartile range) for 13–15 animals in each group. Different from all groups, * $P<0.01$; ** $P<0.001$ (Kruskal–Wallis followed by Dunn's test).

a 0.6 mA foot shock; that shock intensity was used in following experiments. After each trial, the apparatus was cleaned with an ethanol solution (20%). All animals were tested only once.

Analysis of BDNF immunocontent

Mature BDNF protein was assessed using the E-Max ELISA kit (Promega) according to the manufacturer's recommendations. Briefly, hippocampus and parietal cortex were individually homogenized in lysis buffer containing: 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), Igepal (1%), glycerol (10%), 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM sodium vanadate, 0.1 mM EDTA, and 0.1 mM EGTA, and centrifuged for 3 min at 14,000 rpm at 4 °C. Supernatant was diluted (1:5 v/v) in sample buffer and incubated on a 96-well flat-bottom plates previously coated with anti-BDNF monoclonal antibody and blocked with Block and Sample buffer. After sample incubation, plates were incubated with polyclonal anti-human antibody for 2 h and horseradish peroxidase for 1 h. Then color reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm. The standard BDNF curve, ranging from 0 to 500 pg /mL, was performed in each plate.

Folate plasma determination

Plasma folate concentration was measured by an automated chemiluminescence system (ACS: 180, Siemens). The method is based on a competitive immunoassay with acridinium ester-labeled folate in solid phase.

Protein determination

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

Statistical analysis

The step down latencies of inhibitory avoidance task were expressed as medians (interquartile range) and were analyzed by Kruskal–Wallis non-parametric analysis of variance (ANOVA), followed by Dunn's test. Two-way ANOVA, followed by Duncan's test, was used to analyze data from open-field task and BDNF determination. Folate concentration data were analyzed by one-way ANOVA followed by Duncan's *t*-test. Analyses were performed using Statistica v6.0 InStat software. Differences were considered statistically significant if $P<0.05$.

RESULTS

Folic acid pretreatment prevents consolidation impairment in STM and LTM evaluated by step-down inhibitory avoidance task in hyperhomocysteinemic rats

Kruskal–Wallis test followed by post hoc analysis (Dunn's test) revealed that Hcy administration impairs memory consolidation of STM, when the test was performed 1 h after the training session (Fig. 2A; $\chi^2(3)=13.53$; $P<0.01$), and of LTM when the test was performed 24 h after the training session (Fig. 2B; $\chi^2(3)=18.70$; $P<0.001$). Folic acid pretreatment completely prevented the inhibitory avoidance memory impairment elicited by Hcy administration in rats.

In order to confirm the absence of motor effects caused by the treatments we performed the open-field task. Table 1 shows that Hcy, folic acid, and Hcy plus folic acid treatments did not cause any motor alteration in rats, as evaluated by the number of crossing ($F(3,35)=0.008$; $P>0.1$). In addition, the number of rearings was not altered by different treatments (data not showed).

Folic acid pretreatment prevents BDNF reduction in hippocampus of hyperhomocysteinemic rats

In this experiment, we measured BDNF levels in hippocampus and parietal cortex of rats subjected to a pretreatment with folic acid from their 22nd to their 28th day of life and a single Hcy injection immediately after training session on inhibitory avoidance apparatus. Hcy administration reduced BDNF levels, when compared to control group, in hippocampus of rats euthanized 1 h after foot

Table 1. Effect of Hcy, folic acid, and folic acid plus Hcy administration on number of crossings on open-field task

Number of crossings	
Control	62±10
Hcy	64±09
Folic acid	59±06
Folic acid+Hcy	62±13

Data are the number of crossings during 180 s and expressed as mean+SD for 9 to 10 rats in each group. There were no significant difference between groups (two-way ANOVA).

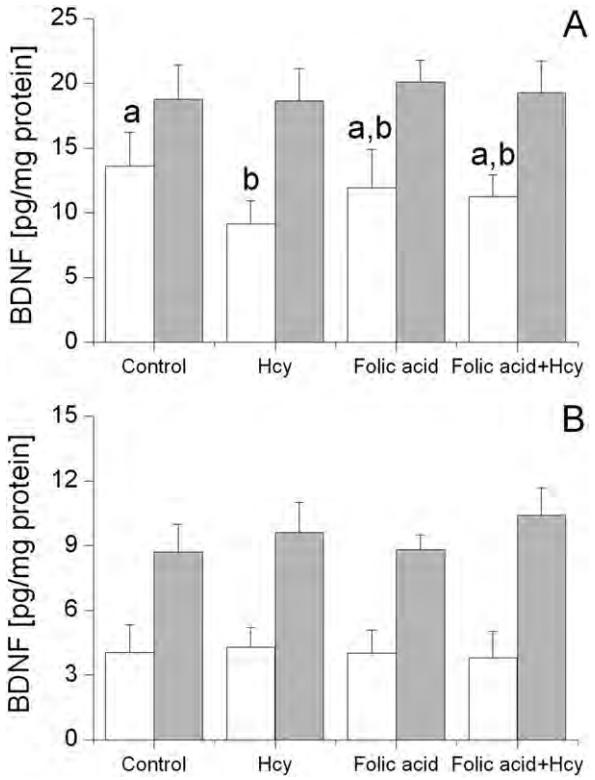

Fig. 3. Effect of folic acid pretreatment on BDNF concentration on hippocampus (A) and parietal cortex (B) of hyperhomocysteinemic rats euthanized 1 h (white bars) or 24 h (gray bars) after the foot shock in inhibitory avoidance apparatus. Results are expressed as mean±SD for five animals in each group. Different from all groups, a≠b, $P<0.05$ (two-way ANOVA followed by Duncan's test).

shock (Fig. 3A; white bars; $t(8)=3.146$; $P<0.05$). Two-way ANOVA followed by Duncan's test showed that folic acid pretreatment prevented Hcy effect on BDNF immunocontent ($F(3,16)=5.400$; $P=0.050$). Conversely, hippocampal BDNF levels were not altered 24 h after Hcy, folic acid, or Hcy plus folic acid treatments (Fig. 3A; gray bars; $F(3,16)=0.396$; $P>0.05$). Fig. 3B shows that Hcy and/or folic acid did not elicit changes in BDNF levels in parietal cortex of rats euthanized 1 h ($F(3,16)=0.164$; $P>0.05$) or 24 h ($F(3,16)=1.917$; $P>0.05$) after training session.

We observed a relative increase in BDNF immunocontent 24 h after the training session in the hippocampus (increased by 40%; $t(8)=-3.092$; $P<0.05$) and parietal cortex (increased by 115%; $t(8)=-5.345$; $P<0.01$), when compared to control rats euthanized 1 h after the foot shock on inhibitory avoidance apparatus.

Folate plasma kinetic curve

In order to verify the kinetic curve of folate plasma levels, we performed a single i.p. administration of folic acid in rats and measured the plasma levels 0 min, 15 min, 1 h, and 3 h later. Fig. 4 shows that folic acid administration increases folate plasma levels up to 1 h after injection, returning to baseline levels after 3 h ($F(3,8)=176.1$; $P<0.001$).

DISCUSSION

In the present study, we showed that Hcy administration immediately after the training session in step-down inhibitory avoidance task impairs consolidation of STM and LTM. Open-field task was utilized as a control for spontaneous activity, and the results showed that none of the tested groups present motor deficit. These results are in agreement with previous reports from our group, showing that a single Hcy administration caused a consistent deficit on acquisition, consolidation, and retrieval of LTM in inhibitory avoidance task (Reis et al., 2002) and chronic hyperhomocysteinemic rats present an impairment in the spatial learning in Morris water maze task (Streck et al., 2004; Matté et al., 2007).

We also investigated the effect of Hcy, administered immediately after training session on inhibitory avoidance apparatus, on BDNF immunocontent in hippocampus and parietal cortex of rats. We used these cerebral structures because of their role in consolidation and other memory mechanisms (Izquierdo et al., 1997; Alonso et al., 2002, 2005). We observed that Hcy reduced BDNF immunocontent in hippocampus, but not in parietal cortex, of rats sacrificed 1 h after the learning stimulus by foot shock. Twenty-four hours after the stimulus, we did not identify any difference in BDNF levels in hippocampus or cerebral cortex. Considering our results and those from literature showing that BDNF level is associated with consolidation mechanisms, we can speculate that the reduction in BDNF levels observed in hippocampus of hyperhomocysteinemic rats could be related to the effect of Hcy on memory consolidation impairment found in STM and/or in LTM.

In this context, Alonso et al. (2002) in a refined study showed that anti-BDNF antibody, infused into the dorsal hippocampus 1 h after training, in inhibitory avoidance apparatus, impaired LTM. The same authors also showed that STM was impaired by infusion of function-blocking anti-BDNF antibody into the dorsal hippocampus prior to, but not immediately or 1 h after, training session. Based on these findings, we suggest that the reduction of BDNF immunocontent in hippocampus caused by Hcy injection,

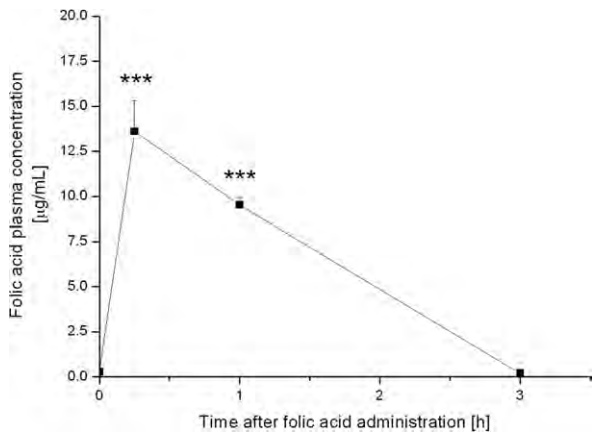

Fig. 4. Folate plasma concentration. Results are expressed as mean±SD for three animals in each group. Different from time 0 h, *** $P<0.001$ (one way ANOVA followed by Duncan's test).

1 h after the learning stimulus, could be associated with the impairment in LTM consolidation. However, we could not discard the effect of BDNF in STM deficit in other time points.

It has been suggested that BDNF reduction appears to be related to oxidative stress (Wu et al., 2004; Kapczinski et al., 2008). Zou and Crews (2006) showed that oxidative stress, as well as glutamate-induced neurotoxicity, reduced BDNF mRNA expression, decreasing CREB DNA binding activity. In addition, Pugazhenthi et al. (2006) reported that lipid peroxidation products decreased the activity of CREB-dependent BDNF promoter in hippocampal neurons. On the other hand, reports show that Hcy provokes oxidative damage by activation of glutamatergic receptors, with consequent reactive species generation (Ho et al., 2003; Jara-Prado et al., 2003; Zieminska and Lazarewicz, 2006) or by autoxidation to homocystine and other disulfides releasing $O_2^{\bullet -}$ and H_2O_2 (Ho et al., 2002; Faraci and Lentz, 2004; Dayal et al., 2004). In this context, we have shown that Hcy induces oxidative damage to hippocampus of rats, increasing lipid peroxidation, and decreasing antioxidant defenses (Streck et al., 2003). The reduction in mature BDNF concentration in the hippocampus during consolidation of memory elicited by Hcy could be a result of oxidative damage, and probably affects negatively BDNF signaling pathways, contributing to the impairment verified in consolidation of memory on inhibitory avoidance task.

Additionally, other aspects could contribute to the memory consolidation impairment elicited by Hcy. Na^+,K^+-ATPase is inhibited by hyperhomocysteinemia in the hippocampus (Streck et al., 2002) and parietal cortex of rats (Matté et al., 2004, 2006, 2007), and there is evidence that this enzyme might play a relevant role in learning and memory (Ng and Gibbs, 1991; Sato et al., 2004; Wyse et al., 2004). It is noteworthy that glutamate receptors play a critical role in memory consolidation in hippocampus, immediately after training, and in parietal cortex 1 h after training (Izquierdo et al., 1997). In addition, there are *in vitro* studies showing that Hcy, in pathological concentrations, can alter glutamate transmission, resulting in excitotoxicity by excessive Ca^{2+} influx and neurodegeneration (Lipton et al., 1997; Ho et al., 2002; Zieminska and Lazarewicz, 2006).

Folic acid has been shown as a safe approach in the prevention and treatment of CNS disorders (Brattstrom et al., 1988; Cook and Hess, 2005; Smith, 2008), such as neurodegenerative diseases (Mattson et al., 2002), cognitive impairments (Kado et al., 2005; Durga et al., 2007), psychiatric diseases (Bottiglieri, 2005), neural tube defects (Blom et al., 2006), and homocystinuria (Mudd et al., 2001). In our study, we showed that folic acid treatment significantly increases plasma folate concentration 15 min and 1 h after injection, returning to control levels after 3 h. Considering that more studies are necessary to establish the effect of folic acid on experimental models of memory deficits, we also evaluated the effect of a pretreatment with this B-vitamin on STM and LTM impairments elicited by Hcy administration. Results showed that folic acid efficiently prevented the deficits in consolidation of memory in hyperhomocysteinemic rats, evaluated by inhibitory avoidance task. To our knowledge, there are a limited number of studies about the mechanisms involved on folic acid effect on memory and cognition. Our findings identified a promising target, showing that the reduction in BDNF levels observed in Hcy-treated rats was partially prevented by folic acid administration.

We have previously shown that folic acid administration prevented spatial memory decline in hyperhomocysteinemic rats, and this effect was independent of the reduction of cerebral and plasma Hcy levels (Matté et al., 2007). Folic acid treatment also prevented Na^+,K^+-ATPase inhibition in parietal cortex of hyperhomocysteinemic rats (Matté et al., 2006, 2007). Consistent with the idea that oxidative stress might prompt cognitive deficit by neurodegeneration (Cantuti-Castelvetri et al., 2000; Cruz et al., 2003; Silva et al., 2004; Butterfield et al., 2006), or by reducing BDNF levels (Wu et al., 2004; Zou and Crews, 2006; Pugazhenthi et al., 2006; Kapczinski et al., 2008), we proposed that folic acid could act as an antioxidant, preventing the memory impairment elicited by Hcy (Matté et al., 2009). In this context, we have recently shown that folic acid chronic treatment prevented the oxidative damage to lipids, proteins, and DNA, as well the reduction in enzymatic and non-enzymatic antioxidant defenses in parietal cortex, induced by Hcy administration in rats (Matté et al., 2009).

Taken together, our results show that Hcy acute administration in rats impaired consolidation of STM and LTM on an inhibitory avoidance task and reduced hippocampal BDNF. Although the fully underlying pathophysiological mechanism by which Hcy induces the memory impairment remains to be determined, we can speculate that the reduction in hippocampal BDNF may be, at least, in part associated with LTM mechanisms. Pretreatment with folic acid prevents STM and LTM deficits and partially prevents the effect of Hcy on BDNF immunocontent in hippocampus of rats, suggesting that this B-vitamin could be a helpful approach to prevent cognitive disorders according to the experimental model used. Further studies are required to assess the entire mechanism by which folic acid acts and its potential therapeutic use for memory impairment prevention in homocystinuric patients.

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PARTE III

IV. DISCUSSÃO

A HCU é uma aminoacidopatia caracterizada bioquimicamente pela deficiência da enzima CBS. Associado aos altos níveis plasmáticos de Hcy, que podem atingir 500 μM, os pacientes apresentam frequentemente retardo mental, crises convulsivas, arteriosclerose, tromboembolismo, alterações oculares, hepáticas e ósseas (Mudd, Levy e Kraus, 2001). A hiper-homocisteinemia também tem sido relatada como fator de risco para o desenvolvimento de doenças cardiovasculares (Clarke et al., 1991; Welch e Loscalzo, 1998; Faraci e Lentz, 2004), além de estar relacionada a desordens neurodegenerativas (Diaz-Arrastia, 2000; Mattson, Kruman e Duan, 2002), psiquiátricas (Sachdev, 2004; Bottiglieri, 2005) e hepáticas (Bosy-Westphal et al., 2001; Adinolfi et al., 2005; Ventura et al., 2005). Streck e cols. (2002a) desenvolveram um modelo experimental de hiper-homocisteinemia, através da administração subcutânea diária de Hcy, a fim de obter níveis plasmáticos similares aos encontrados nos pacientes homocistinúricos. Esse modelo tem início no 6º dia de vida pós-natal dos ratos, pois o desenvolvimento cerebral dos animais nesse dia é similar ao de um humano neonato (Clancy et al., 2007).

A análise dos estudos realizados até o presente momento indica que a toxicidade da Hcy, em diferentes tecidos e tipos celulares, parece estar associada aos seguintes temas: (1) desequilíbrio das reações de metilação celular (Troen, 2005; Jamaluddin, Yang e Wang, 2007); (2) excitotoxicidade, via ativação de receptores glutamatérgicos (Lipton et al., 1997; Ho et al., 2002; Troen, 2005; Zieminska e Lazarewicz, 2006); (3) estresse oxidativo (Mudd, Levy e Kraus, 2001; Ho et al., 2002; Wyse et al., 2002; Ho et al., 2003; Streck et al., 2003b; Faraci e Lentz, 2004); e (4) disfunção mitocondrial e depleção energética (Kruman et al., 2000; Streck et al., 2003a). Nesta tese investigamos o efeito da Hcy sobre parâmetros comportamentais, avaliando as memórias espacial e

aversiva; e bioquímicos, analisando parâmetros de estresse oxidativo, do metabolismo energético e do sistema glutamatérgico em ratos Wistar.

Considerando que o ácido fólico participa do metabolismo da Hcy, como precursor da coenzima N^5-metiltetraidrofolato (Fowler, 1997; Brosnan et al., 2004; Finkelstein, 2007); e possui um emergente papel como antioxidante (Doshi et al., 2001; Joshi et al., 2001; Au-Yeung et al., 2006); esta tese também abrangeu a avaliação do efeito do ácido fólico sobre as alterações comportamentais e bioquímicas causadas pela administração de Hcy. Nesse contexto, alguns trabalhos da literatura mostram que essa vitamina do complexo B tem sido empregada na prevenção de defeitos no fechamento do tubo neural (Finglas et al., 2003; Blom et al., 2006), na epilepsia (Morrell, 2002), na prevenção de doenças neurodegenerativas (Mattson, Kruman e Duan, 2002; Mattson e Shea, 2003) e psiquiáticas (Bottiglieri, 2005; Miller, 2008). Apesar da sua utilização clínica, pouco se sabe sobre os efeitos e o mecanismo de ação do ácido fólico.

Nossos estudos iniciaram avaliando o efeito da administração crônica de Hcy sobre alguns parâmetros de estresse oxidativo em córtex parietal de ratos. Mostramos que a hiper-homocisteinemia reduz as defesas antioxidantes enzimáticas, representadas pela CAT e pela GPx, e não-enzimáticas, medidas através da técnica do TRAP. As enzimas antioxidantes pertencem a um sistema coordenado de eliminação das ERO (Halliwell e Gutteridge, 2007). A administração de Hcy pode resultar no acúmulo de peróxido de hidrogênio, desde que as duas enzimas responsáveis por sua remoção, CAT e GPx, estão inibidas em córtex parietal. O radical hidroxil, que tem como precursor o peróxido de hidrogênio, pode ser responsável pela quebra de fita simples e dupla de DNA (Halliwell e Gutteridge, 2007). As defesas antioxidantes não-enzimáticas também se encontraram reduzidas no plasma de ratos tratados cronicamente com Hcy. Por outro lado, as defesas antioxidantes enzimáticas apresentaram um aumento significativo nas

atividades da SOD e da CAT em sangue de ratos hiper-homocisteinêmicos. A Hcy pode regular a expressão e a degradação de algumas enzimas, tais como SOD e GPx, resultando em aumento ou redução na sua atividade antioxidante (Stern et al., 2004; Sharma et al., 2006). Em adição, o aumento na atividade da SOD resulta no aumento do seu produto, peróxido de hidrogênio, o que pode estimular a atividade da enzima seguinte nessa via de eliminação das ERO, a CAT (Halliwell e Gutteridge, 2007).

Também verificamos um aumento proeminente no dano ao DNA, verificado pelo ensaio cometa em córtex parietal e sangue de ratos tratados cronicamente com Hcy. As alterações verificadas nas defesas antioxidantes cerebrais e periféricas descritas acima podem contribuir para o aumento na quantidade de espécies reativas, tais como radical hidroxil e peróxidos lipídicos, que são os principais responsáveis pelo dano ao DNA, promovendo quebras de fitas simples e duplas, bem como modificações oxidativas nas bases do DNA (Halliwell, 2001; Higuchi, 2003). Em adição, o DNA possui metais agregados, tais como ferro e cobre, o que pode facilitar a formação de radicais hidroxil no sítio das quebras de fita (Higuchi, 2003). Corroborando com esses dados, estudos prévios mostram que a Hcy *in vitro* induz estresse oxidativo, induzindo a peroxidação lipídica e reduzindo as defesas antioxidantes não-enzimáticas, em córtex parietal (Matté et al., 2004), assim como no hipocampo (Streck et al., 2003b). Wyse e cols. (2002) previamente mostraram que a administração aguda de Hcy reduz as defesas antioxidantes enzimática (CAT) e não-enzimática em hipocampo de ratos. O pré-tratamento com as vitaminas E & C preveniu tal efeito. A geração de espécies reativas pela Hcy, diretamente via auto-oxidação (Mudd, Levy e Kraus, 2001; Dayal et al., 2004; Faraci e Lentz, 2004) ou ainda indiretamente, via ativação de receptores glutamatérgicos ionotrópicos e metabotrópicos em neurônios (Kim e Pae, 1996; Lipton et al., 1997; Ho et al., 2003; Jara-Prado et al., 2003; Zieminska e Lazarewicz, 2006),

bem como em células do sistema imune periférico (Boldyrev, Carpenter e Johnson, 2005) podem contribuir para o dano ao DNA observado no presente estudo.

Diversos estudos têm demonstrado a potencialidade *in vitro* da Hcy em causar alterações oxidativas, seguidas de dano ao DNA e morte celular por apoptose. Kruman e cols. (2000), utilizando culturas primárias de neurônios hipocampais de ratos, demonstraram que a Hcy induz morte celular via apoptose, a partir da concentração de 0,5 μM, como consequência do estresse oxidativo e dano ao DNA. Em oposição, culturas de astrócitos parecem não ser afetadas pela Hcy, mesmo em concentrações na ordem de mM. Em neurônios, a Hcy depleta os níveis celulares de ATP, via ativação da PARP, uma enzima de reparo do DNA, que é ativada exclusivamente em situações de quebra de fita do DNA (Kruman et al., 2000), tais como as detectadas pelo ensaio cometa em nosso estudo (Matté et al., 2009a). Além disso, a Hcy promove a ativação da caspase-3 e um aumento na expressão de p53, ambos associados à morte celular apoptótica. A incubação com Hcy reduz o potencial de membrana mitocondrial, que é precedido pelo aumento na produção de ERO em neurônios hipocampais em cultura. Em adição, concentrações subtóxicas de Hcy induzem morte celular apoptótica quando incubadas concomitantemente com metais (Fe^{2+} e Cu^{2+}), produtos de peroxidação lipídica (4-hidroxinonenal - HNE), glutamato e cainato, sugerindo um papel relevante do estresse oxidativo e da excitotoxicidade glutamatérgica na neurodegeneração induzida pela Hcy (Kruman et al., 2000). A Hcy (10-250 μM) promoveu o influxo maciço de Ca^{2+} em neurônios corticais de ratos em cultura, via receptores NMDA e secundariamente via receptores metabotrópicos, assim como induziu a geração de ERO (peróxidos) e promoveu apoptose. Tais efeitos foram parcialmente prevenidos pela coincubação com MK801, um inibidor de canais NMDA, indicando que a citotoxicidade da Hcy é parcialmente dependente dos receptores glutamatérgicos (Ho et

al., 2002). Perifericamente, o dano ao DNA observado em nosso estudo também foi demonstrado por Picerno e cols. (2007), que descrevem a ação da Hcy sobre linfócitos humanos em cultura. A incubação por 48h na presença de 1 mM de Hcy impediu a entrada das células no ciclo celular quando estimuladas por concanavalina A, e promoveu apoptose em linfócitos, associada à ativação da caspase-3 e aumento na frequência de micronúcleos.

O tratamento concomitante com ácido fólico resultou na prevenção total das alterações verificadas nos parâmetros de estresse oxidativo induzidas pela administração de Hcy. Acreditamos que o papel do ácido fólico como modulador do estresse oxidativo, devido a atividade antioxidante demonstrada por essa molécula e descrita em detalhes na introdução desse trabalho (Joshi et al., 2001; Au-Yeung et al., 2006) possa estar correlacionada com a prevenção dos parâmetros de estresse oxidativo avaliados nesse trabalho. Em adição, Patro e cols. (2006) descrevem a ação do ácido fólico como um modulador do estado redox do ferro, promovendo a oxidação do Fe^{2+} à Fe^{3+}, impedindo a ação oxidativa desse metal através da reação de Fenton. Por outro lado, diversos trabalhos têm demonstrado a ação genômica do ácido fólico, estabilizando o DNA, além de sua participação na síntese de nucleotídeos desse ácido nucléico. Nesse contexto, baixos níveis de ácido fólico reduzem a concentração das coenzimas derivadas dessa vitamina, prejudicando a síntese de dTMP a partir de dUMP. A alta disponibilidade desse nucleotídeo promove a incorporação errática de uracila no DNA, ao invés de timina; o que pode ser responsável por mutações de ponto, quebras de fita e quebras cromossômicas, bem como a formação de micronúcleos (Fenech, 2001; Fenech e Crott, 2002).

Corroborando com nossos dados, Ho e cols. (2003) cultivaram neurônios corticais provenientes de roedores Murinos na presença e ausência de folato. A

deprivação de folato, e concomitante aumento nos níveis de Hcy, promoveram um aumento no influxo de Ca^{2+}, detectado pelo indicador de cálcio Fluo-3, associado à geração de ERO, medidas pelo método do diacetato de diclorofluoresceína, bem como um incremento na externalização de fosfoserina e morte celular apoptótica. Quando a Hcy foi adicionada ao meio de cultura, observou-se uma exacerbação dos efeitos da deprivação de folato, enquanto a adição de um inibidor da síntese de Hcy, de antioxidantes (*N*-acetilcisteína e vitamina E) ou de MK801 preveniram os efeitos da deprivação de folato, indicando um papel relevante do estresse oxidativo e da excitotoxicidade glutamatérgica associados à injúria induzida pela deprivação de folato e consequente aumento dos níveis de Hcy em cultura de neurônios.

O segundo capítulo da presente tese estendeu a avaliação de parâmetros de estresse oxidativo para o fígado de ratos submetidos à administração crônica de Hcy. Nossos dados revelam que o tecido hepático também é suscetível ao estresse oxidativo induzido pela Hcy, demonstrado através da redução das defesas antioxidantes enzimática, representada pela atividade da CAT, e não-enzimática, medida pelos ensaios do TRAP e do TAR. A administração crônica de Hcy também promoveu um aumento na quimioluminescência e no TBARS, bem como uma redução no conteúdo de grupamentos tióis em fígado de ratos. Apesar das alterações bioquímicas observadas em fígado de ratos hiper-homocisteinêmicos, as atividades das aminotransferases, alanina aminotransferase (ALT) e aspartato aminotransferase (AST), não foram significativamente alteradas no fígado e no plasma desses animais.

A redução no conteúdo de sulfidrilas observada em nosso estudo pode estar relacionada com a redução de antioxidantes tiólicos, tais como a GSH, o que está de acordo com a redução no TRAP e no TAR observados; mas também pode refletir uma redução de grupos tióis protéicos. Nesse sentido, a oxidação de proteínas pode resultar:

(1) da formação de aductos entre os resíduos de aminoácidos e os produtos de peroxidação lipídica, tais como HNE e MDA, identificado na técnica de TBARS (Houglum et al., 1990; Comporti, 1993), e/ou (2) da oxidação direta das proteínas por espécies reativas, tais como peroxinitrito, que pode resultar da ação da Hcy (Radi et al., 1991).

Woo e cols. (2006) recentemente demonstraram que ratos hiper-homocisteinêmicos, alimentados com uma dieta rica em Met durante quatro semanas, apresentaram dano hepático, resultado do aumento de superóxido via ativação da NAD(P)H oxidase, e da formação de aductos protéicos de nitrotirosina derivados da ação do peroxinitrito. Também foi observado um incremento na peroxidação lipídica, medida através do conteúdo de malondialdeído (MDA). As atividades das enzimas antioxidantes SOD e CAT estavam reduzidas no tecido hepático desses animais, enquanto a atividade da GPx permaneceu inalterada. Esses dados revelam que a hiper-homocisteinemia induziu estresse oxidativo no fígado de ratos via aumento na produção de ERO e consequente peroxidação lipídica, associada a uma redução na atividade antioxidante, o que está de acordo com os resultados obtidos no capítulo II da presente tese (Matté et al., 2009b). O estudo de Woo e cols. (2006) mostrou ainda um aumento na atividade sérica das aminotransferases, refletindo a injúria hepática, o que não foi observado em nosso trabalho. Em adição, o modelo experimental de hiper-homocisteinemia, baseado na deprivação de ácido fólico, promoveu o aumento do TBARS em fígado de ratos (Huang et al., 2001; Chanson et al., 2007). A atividade da CAT hepática não foi alterada nesse modelo de estudo, entretanto foi observada uma correlação negativa entre os níveis plasmáticos de Hcy e a atividade dessa enzima antioxidante (Chanson et al., 2007).

Ji e Kaplowitz (2004) revisaram o papel da Hcy na injúria hepática e indicam três maiores pilares responsáveis pelos efeitos desse aminoácido: (1) estresse oxidativo, (2) estresse derivado do retículo endoplasmático, e (3) ativação de fatores pró-inflamatórios. Nossos dados suportam a hipótese de dano hepático como consequência do estresse oxidativo induzido pela administração crônica de Hcy. Sabe-se que o estresse oxidativo está associado a importantes modificações morfológicas no tecido hepático (Poli, 1993; Poli e Parola, 1997). Nesse contexto, realizamos a avaliação histológica do tecido hepático de ratos submetidos à administração crônica de Hcy. A coloração de hematoxilina/eosina não demonstrou alterações morfológicas ou esteatose em preparações do tecido hepático de ratos tratados com Hcy, entretanto pode-se observar um significativo infiltrado inflamatório, mais evidente no tecido conjuntivo próximo ao espaço porta e contornando os grandes vasos. Woo e cols. (2008) induziram hiper-homocisteinemia, através da suplementação com metionina, e observaram o aumento na expressão e na síntese hepática da proteína quimiotática de monócitos 1 (MCP-1), o que pode estar relacionado à migração de leucócitos observada em nosso estudo. Esses dados estão de acordo com a proposição de Ji e Kaplowitz (2004), que reportou o efeito da Hcy sobre mediadores inflamatórios, aumentando os níveis de NFκB e de uma série de interleucinas (IL) pró-inflamatórias no fígado.

A coloração de Picrosírius revelou um aumento significativo de fibras de colágeno em torno dos hepatócitos, assim como no tecido conjuntivo próximo ao espaço porta e contornando os grandes vasos, o que caracteriza a presença de fibrose hepática. A deposição de colágeno extracelular é resultante do desequilíbrio entre produção e degradação da matriz. O dano hepático promove a ativação de células especializadas na produção da matriz extracelular, as células estreladas hepáticas, resultando no acúmulo de colágeno extracelular, que é responsável pelo aspecto fibroso do tecido afetado

(Henderson e Forbes, 2008). Recentemente, Zou e cols. (2009) mostraram que a Hcy (0,05-0,5 mM) promove a ativação das células estreladas hepáticas isoladas de fígado de ratos, convertendo as células no estado quiescente para uma morfologia de miofibroblastos, responsáveis pela fibrose hepática. A ativação das células estreladas é dependente da geração de ERO, que ocorre num primeiro momento independentemente da atividade da NAD(P)H oxidase e posteriormente de uma forma condicionada a ativação dessa enzima. A ativação da NAD(P)H oxidase é mediada pelas ERO geradas pela Hcy, seguida pela ativação da via das MAPKs (Zou et al., 2009). As células estreladas hepáticas ativadas adquirem um fenótipo secretor de citocinas pró-inflamatórias, além de aumentar a expressão de moléculas de adesão, o que pode estar associado ao recrutamento de células inflamatórias, evidenciado pelo nosso estudo (Kisseleva e Brenner, 2007; Friedman, 2008). Por outro lado, não podemos descartar a possibilidade de alterações na degradação da matriz extracelular, que é desempenhada pelas metaloproteinases (MMP) (Friedman, 2008). A Hcy aumenta a expressão e a síntese hepáticas do inibidor de MMP-1 (TIMP-1) em camundongos CBS-deficientes (Robert et al., 2005) e em cultura de hepatócitos e células estreladas hepáticas (Torres et al., 1999).

Em concordância com nossos resultados, camundongos hiper-homocisteinêmicos CBS-deficientes apresentaram infiltrado inflamatório mononuclear em torno dos vasos hepáticos, esteatose, bem como filamentos fibrosos envolvendo os hepatócitos. Além disso, esses animais apresentaram um aumento na expressão de pró-colágeno α tipo I e de citocinas pró-inflamatórias, tais como fator de necrose tumoral α (TNF-α) e IL-6, aliado à ativação das células estreladas hepáticas. Essas alterações foram acompanhadas por aumento da peroxidação lipídica e da oxidação protéica,

medida pelo conteúdo de carbonilas, em camundongos CBS-deficientes (Robert et al., 2005).

A coloração de ácido periódico de Schiff (PAS) identificou uma redução no conteúdo de glicogênio ou glicoproteínas no fígado de ratos tratados com Hcy. Mais estudos são necessários a fim de desvendar a relevância desses dados e os mecanismos relacionados a esse fenômeno.

A partir do capítulo III passamos a avaliar o efeito da Hcy sobre alguns parâmetros de metabolismo energético e do sistema glutamatérgico em córtex parietal, bem como sobre parâmetros comportamentais.

O estresse oxidativo está diretamente associado à mitocôndria, o sítio celular de maior produção de ERO, o que pode promover a disfunção mitocondrial observada em doenças neurodegenerativas (Beal, 2000; Sullivan e Brown, 2005; Zeviani e Carelli, 2007). Na mitocôndria a peroxidação lipídica, induzida pelas ERO, atinge a cardiolipina, um glicerofosfolipídio presente na membrana mitocondrial interna, onde está associada ao citocromo *c* e aos complexos da cadeia transportadora de elétrons (Muralikrishna Adibhatla e Hatcher, 2006; Pope, Land e Heales, 2008; Addabbo, Montagnani e Goligorsky, 2009). Estudos prévios de nosso grupo de pesquisa mostram que a administração crônica de Hcy inibe a atividade da SDH e da COX, bem como a captação de glicose e a atividade do ciclo de Krebs em hipocampo de ratos, promovendo uma substancial disfunção no metabolismo mitocondrial (Streck et al., 2003a). Nesse contexto, avaliamos o efeito agudo e crônico da Hcy sobre algumas enzimas chaves no metabolismo energético em córtex parietal. A enzima CK não apresentou alteração significativa na atividade das isoformas mitocondrial e citosólica em córtex parietal de ratos submetidos à administração aguda de Hcy e sacrificados 1 hora após. A SDH e o complexo II da cadeia transportadora de elétrons também não

sofreram alteração na sua atividade, entretanto a atividade da COX apresentou uma inibição significativa em córtex parietal de ratos sacrificados 1 hora após uma única administração de Hcy. Doze horas após, a atividade dessa enzima retornou aos níveis de controle.

A COX não parece ser uma fonte importante de ERO, que são geradas principalmente por redução monoeletrônica do oxigênio por elétrons provenientes do citocromo *c*, e dos complexos I, II e III da cadeia transportadora de elétrons. Entretanto, a inibição da COX e consequente redução da transferência de elétrons na cadeia transportadora mitocondrial pode estar associada à geração de ERO (Kowaltowski et al., 2009). Resultados recentes de Linnebank e cols. (2006) mostraram que a Hcy *in vitro* reduz a viabilidade celular em cultura primária de neurônios granulares cerebelares de ratos, bem como em culturas de linhagens de células de feocromocitoma de rato (PC12) e de neuroblastoma humano (SHSY-5Y). O ensaio de TUNEL confirmou o tipo de morte celular como apoptótica. O complexo I não apresentou alteração na atividade, enquanto a COX foi inibida pela incubação com Hcy, em um mecanismo possivelmente envolvendo a interação com os sítios de Cu^{2+} presentes na enzima. A inibição da COX ocorreu concomitantemente com a geração de ERO, detectadas pelo ensaio do diacetato de diclorofluoresceína, sendo significativa em concentrações de Hcy maiores que 50 μM. Em adição, Delgado e cols. (2006) mostraram que a administração de malation, um organofosforado, que induz a formação de superóxido, promove a peroxidação lipídica associada à inibição da COX em hipocampo de ratos. A atividade do complexo II não foi alterada nessa estrutura cerebral.

Tendo em mente que os complexos da cadeia respiratória estão inseridos na membrana mitocondrial interna, a peroxidação lipídica pode ser um fator relevante nas alterações da atividade dessas enzimas (Delgado et al., 2006; Sen et al., 2006; Pope,

Land e Heales, 2008). A cardiolipina, que perfaz 25% do total de lipídios na membrana mitocondrial, aparece associada à COX, que tem sua atividade reduzida em situações de perda de cardiolipina (Pope, Land e Heales, 2008). A administração aguda de Hcy foi capaz de aumentar a quimioluminescência, 30 e 60 min. após a injeção de Hcy. O ataque de ERO às membranas, além de alterar a composição e a permeabilidade das mesmas, ainda promove a liberação de produtos, tais como MDA, HNE e acroleína, que potencialmente atacam biomoléculas tais como DNA e proteínas, propagando o dano das ERO (Halliwell, 2001; Halliwell, 2006; Sen et al., 2006). Nesse contexto, Sen e cols. (2006) mostraram que mitocôndrias cerebrais de ratos isoladas submetidas a um meio pró-oxidativo, contendo ferro e ascorbato, apresentaram um aumento no conteúdo de MDA, associado à perda de cardiolipina, bem como do potencial de membrana mitocondrial. Esses parâmetros foram alterados por um mecanismo dependente da peroxidação lipídica, desde que foram prevenidos pela adição de um antioxidante que impede a lipoperoxidação, o di-terc-butil metil fenol (BHT). Em adição, a peroxidação lipídica pode promover a liberação do citocromo *c* pela cardiolipina, o que está associado à indução de morte celular apoptótica (Addabbo, Montagnani e Goligorsky, 2009).

Posteriormente, investigamos se o ácido fólico poderia prevenir o efeito da Hcy sobre a atividade da COX, e caso confirmássemos nossa hipótese, verificar se o estresse oxidativo está envolvido nesse efeito. Nossos dados mostram que o pré-tratamento com ácido fólico durante uma semana preveniu completamente a inibição da COX induzida pela administração aguda de Hcy. Acreditamos que a Hcy atue sobre a COX por um mecanismo dependente do estresse oxidativo, desde que o pré-tratamento com as vitaminas E & C preveniu a inibição dessa enzima. Nesse sentido, o ácido fólico previne a peroxidação lipídica, medida 30 e 60 min após a administração de Hcy,

sugerindo que essa vitamina possa atuar como antioxidante prevenindo os efeitos deletérios da Hcy.

O efeito da administração crônica de Hcy, do 6º ao 28º dia de vida dos animais, sobre o metabolismo energético também foi avaliado. Nossos dados mostraram que o tratamento crônico não promoveu alteração significativa nas atividades da CK, da SDH e do complexo II da cadeia respiratória, similar aos resultados obtidos para a administração aguda de Hcy. Entretanto a hiper-homocisteinemia crônica aumentou a atividade da COX em córtex parietal de ratos. Interessantemente, a administração crônica de Hcy também induz o aumento na peroxidação lipídica em córtex parietal, medido pela técnica de TBARS (Matté et al., 2007). Nesse contexto, Huertas et al. (1992) mostraram que a peroxidação lipídica pode induzir um aumento na expressão dessa enzima, ao menos em coração de ratos. Austin et al. (1998) mostraram que a expressão do gene mitocondrial da COXIII/ATPase 6,8 está aumentada, de forma concentração e tempo-dependentes, na linhagem celular de megacariócitos DAMI, quando incubada com Hcy em concentrações de até 1 mM. O autor sugere que esse aumento nos níveis de COXIII/ATPase 6,8 é dependente do aumento de peróxido de hidrogênio e pode estar correlacionado com a depleção energética resultante da utilização de ATP nas vias de metabolização e eliminação celular desse aminoácido. O aumento do mRNA da COX também foi encontrado em cérebros de pacientes portadores de Alzheimer (Manczak et al., 2004), podendo resultar no aumento da atividade da enzima, o que está associado ao aumento da produção de ERO e à neurodegeneração (Kadenbach et al., 2004). Nossos resultados mostram a ativação da COX em córtex parietal de ratos tratados cronicamente com Hcy, bem como um aumento nos índices de peroxidação lipídica (Matté et al., 2007), dano oxidativo ao DNA, e redução das defesas antioxidantes (Matté et al., 2009a). Apesar de existir uma

forte correlação entre estresse oxidativo e disfunção mitocondrial, não existe um consenso entre causa e efeito.

A produção de ERO parece ser essencial para a ação da Hcy, que na concentração de 100 μM promove a ativação de NFκB e o resultante aumento na expressão da subunidade III da COX em células endoteliais. A adição de antioxidantes previne a superexpressão do mRNA da COX, bem como a ativação do fator de transcrição, sugerindo a participação do estresse oxidativo no mecanismo de ação da Hcy (Perez-de-Arce, Foncea e Leighton, 2005). Isto posto, nosso próximo objetivo foi verificar o efeito da administração concomitante de ácido fólico em ratos cronicamente tratados com Hcy. O ácido fólico preveniu o aumento da COX em córtex parietal de ratos hiper-homocisteinêmicos. As evidências de que o estresse oxidativo é importante no mecanismo de dano mitocondrial exercido pela Hcy, permite sugerir que o ácido fólico possa atuar como antioxidante prevenindo tais efeitos (Joshi et al., 2001; Au-Yeung et al., 2006; Patro et al., 2006; Matté et al., 2007; Matté et al., 2009a).

Uma das hipóteses mais investigadas a respeito do mecanismo de neurotoxicidade da Hcy contempla sua ação sobre o sistema glutamatérgico. Diversos estudos têm mostrado o papel da Hcy sobre os receptores ionotrópicos e metabotrópicos do glutamato, onde ela poderia atuar como um agonista superativando esses receptores, induzindo o influxo de Ca^{2+}, o que pode resultar na geração de radicais livres e morte celular (Lipton et al., 1997; Jara-Prado et al., 2003; Zieminska, Stafiej e Lazarewicz, 2003; Zieminska e Lazarewicz, 2006). Essa hipótese excitotóxica valoriza o papel dos receptores glutamatérgicos em detrimento dos transportadores de glutamato, os quais têm ganhado espaço nos mecanismos fisiopatológicos de algumas doenças neurodegenerativas (Sheldon e Robinson, 2007). O capítulo IV da presente tese mostrou que as administrações aguda e crônica de Hcy inibem a captação de glutamato em

córtex parietal de ratos. Ratos de 6 dias de vida receberam uma única administração de Hcy e a captação de glutamato permaneceu significativamente reduzida depois de 1, 12 horas, 7 e 30 dias. Por outro lado, quando administrada a ratos de 29 dias de vida, a Hcy foi capaz de inibir a captação de glutamato por apenas algumas horas (1 e 8 h), voltando aos níveis de controle 12 horas após a administração. Esses dados indicam que o SNC em desenvolvimento é mais suscetível aos efeitos neurotóxicos da Hcy, tendo em vista que 30 dias após a administração de Hcy ainda observam-se os efeitos sobre a captação de glutamato, enquanto ratos de 29 dias sofreram um efeito passageiro e reversível da Hcy, recuperando a atividade normal dos transportadores em 12 horas. Já o tratamento crônico com Hcy, do 6º ao 28º dia de vida resultou na inibição da captação de glutamato em córtex parietal de ratos sacrificados 12 horas, 1, 7 e 30 dias após a última administração de Hcy.

Considerando que os transportadores gliais, GLAST e GLT-1, contribuem majoritariamente para a retirada de glutamato da fenda sináptica, e que a Hcy reduz a captação de glutamato, medimos o imunoconteúdo desses transportadores em córtex parietal de ratos tratados cronicamente com Hcy. Observamos que a hiper-homocisteinemia crônica reduziu os níveis de ambos transportadores de glutamato. Enquanto o GLAST possui uma distribuição mais pronunciada em células gliais cerebelares, o GLT-1 é detectado em células gliais em todo o cérebro, sendo responsável pela captação de aproximadamente 90% do glutamato no tecido adulto (Gegelashvili e Schousboe, 1997; Maragakis e Rothstein, 2004). A expressão dos transportadores varia durante o desenvolvimento, no nascimento GLT-1 está praticamente ausente, sendo detectado a partir do 6º dia de vida e aumentando sua expressão coincidentemente com a sinaptogênese, alcançando níveis de adulto em torno do 35º dia de vida do rato. A expressão de GLAST é muito semelhante, aumentando em

paralelo com a de GLT-1, entretanto o GLAST pode ser detectado precocemente, já no momento do nascimento do rato (Furuta, Rothstein e Martin, 1997; Ullensvang et al., 1997). A redução no imunoconteúdo dos transportadores gliais de glutamato provavelmente é responsável pela inibição da captação de glutamato observada nos ensaios bioquímicos, ao menos em ratos submetidos ao tratamento crônico. A redução da captação de glutamato promove o acúmulo desse aminoácido excitatório na fenda sináptica, podendo levar à superativação dos receptores glutamatérgicos e consequente excitotoxicidade (Maragakis e Rothstein, 2004; Sheldon e Robinson, 2007), o que pode estar associado à neurotoxicidade da Hcy e resultante neurodegeneração (Kruman et al., 2000; Ho et al., 2002; Mattson, Kruman e Duan, 2002; Ho et al., 2003).

A inibição na captação de glutamato possivelmente é resultado da redução dos níveis de transportadores, entretanto outros fatores podem estar associados. Existem evidências mostrando a ação inibitória de produtos de peroxidação lipídica e radicais livres, que podem atuar diretamente sobre os grupos tiólicos dos transportadores (Volterra et al., 1994; Gegelashvili e Schousboe, 1997; Maragakis e Rothstein, 2004; Sheldon e Robinson, 2007). Nesse contexto, mostramos nos capítulos anteriores que as administrações aguda e crônica de Hcy promovem estresse oxidativo, incluindo peroxidação lipídica em córtex parietal de ratos (Matté et al., 2007; Matté et al., 2009a). Além disso, a disfunção mitocondrial induzida pela administração de Hcy, pode induzir o aumento na produção de ERO, e assim afetar a atividade dos transportadores de glutamato (capítulo III). As ERO, tais como superóxido e peróxido de hidrogênio, também podem ser formadas como resultado da auto-oxidação da Hcy (Mudd, Levy e Kraus, 2001; Dayal et al., 2004; Faraci e Lentz, 2004), ou via ativação de receptores glutamatérgicos (Kim e Pae, 1996; Lipton et al., 1997; Ho et al., 2003; Jara-Prado et al., 2003; Zieminska e Lazarewicz, 2006).

A Na^+,K^+-ATPase possui um papel essencial na manutenção do gradiente de Na^+, fundamental para a captação de glutamato (Danbolt, 2001; Squire et al., 2003; Magistretti, 2006). A inibição dessa enzima pela Hcy já foi demonstrado em estudos *in vitro* (Matté et al., 2004) e *in vivo* (Matté et al., 2006; Matté et al., 2007) em córtex parietal de ratos, o que pode resultar num prejuízo na captação de glutamato. A perda do gradiente de Na^+, também pode induzir a reversão do transporte de glutamato promovendo um acúmulo maior de glutamato na fenda sináptica (Danbolt, 2001; Maragakis e Rothstein, 2004; Sheldon e Robinson, 2007; Nicholls, 2008).

Nossos dados sugerem que a inibição da captação de glutamato observada em nosso estudo, prejudica a retirada do glutamato da fenda sináptica, prolongando sua ação, o que pode resultar em superativação dos receptores glutamatérgicos pelo seu agonista natural. A ativação dos receptores promove o influxo de Ca^{2+}, que acumula na mitocôndria, podendo resultar no aumento das ERO (Nicholls, 2008). A inibição dos transportadores GLAST e GLT-1, que pode ser resultado da redução na expressão, do dano oxidativo ou da perda do gradiente de Na^+, completa um circuito de excitotoxicidade glutamatérgica (Volterra et al., 1994; Maragakis e Rothstein, 2004; Sheldon e Robinson, 2007).

Nesse estudo relacionamos os efeitos neurotóxicos da Hcy e neuroprotetores do ácido fólico com parâmetros de estresse oxidativo, de metabolismo energético e do sistema glutamatérgico. Relatos da literatura sugerem que alterações oxidativas (Cantuti-Castelvetri, Shukitt-Hale e Joseph, 2000; Cruz, Almaguer Melian e Bergado Rosado, 2003; Serrano e Klann, 2004; Silva et al., 2004; Butterfield et al., 2006), disfunção mitocondrial (Beal, 2000; Sullivan e Brown, 2005; Zeviani e Carelli, 2007; Yang et al., 2008), e prejuízo na captação de glutamato (Maragakis e Rothstein, 2004; Carvalho et al., 2006; Rotta et al., 2008) estão associados a alterações no aprendizado e

memória nas tarefas do labirinto aquático de Morris e esquiva inibitória. Nesse contexto, os dois últimos capítulos da presente tese investigam o efeito da Hcy sobre a memória espacial e aversiva em ratos, bem como o efeito protetor do ácido fólico sobre as alterações cognitivas induzidas pela administração de Hcy, desde que essa vitamina mostrou um potencial benefício em todos os parâmetros bioquímicos apresentados nessa tese.

A administração crônica de Hcy promove um prejuízo na memória espacial de ratos, determinada na tarefa do labirinto aquático de Morris. Streck e cols. (2004) previamente mostraram que a Hcy reduz o número de cruzamentos sobre o local da plataforma, na sessão de teste da memória de referência, bem como induz uma alteração significativa na memória de trabalho. Em concordância, nossos resultados mostram que a administração crônica de Hcy, do 6º ao 28º dia de vida dos animais, promoveu um déficit na memória de referência espacial, observada através do aumento na latência para cruzar o local da plataforma e da redução do número de cruzamentos sobre o local da plataforma na sessão de teste. Em adição, a memória espacial de trabalho também foi afetada pela hiper-homocisteinemia crônica. Utilizamos como controles experimentais a velocidade de natação dos ratos e o desempenho motor na tarefa de campo aberto, e nenhuma alteração significativa foi identificada em nenhum dos grupos experimentais, sugerindo que os efeitos observados na tarefa do labirinto aquático de Morris não está condicionada a desordens motoras que pudessem ser induzidas pelos tratamentos. Recentemente, Troen e cols. (2008) mostraram que camundongos C57BL6/J hiper-homocisteinêmicos alimentados com uma dieta deficiente em vitaminas do complexo B apresentaram prejuízo na memória espacial na tarefa de Water maze, associado à redução na vascularização hipocampal. Troen e cols. (2006) reportaram que camundongos hiper-homocisteinêmicos ApoE-deficientes apresentaram prejuízo na

memória espacial, avaliada através do labirinto aquático, associado a alterações nos processos de metilação celular. Bernardo e cols. (2007) também descrevem um déficit na memória espacial de referência, testada no Water maze, em camundongos que super-expressam a proteína precursora amilóide, nos quais foi induzida hiper-homocisteinemia através de uma dieta restrita em ácido fólico, colina e Met; e suplementada com Hcy. Em adição, Baydas e cols. (2008) induziram hiper-homocisteinemia em ratas grávidas e observaram um déficit na memória espacial nos ratos descendentes dessa gestação. Esses estudos corroboram com nossos dados, evidenciando o déficit mnemônico induzido pela Hcy em diversos modelos animais de hiper-homocisteinemia, associada com outras situações patológicas ou não. Entretanto os mecanismos pelos quais a Hcy altera a memória espacial ainda não foram esclarecidos, e possivelmente um efeito complexo e multifatorial seja responsável pela ação dessa neurotoxina.

A memória avaliada por essa tarefa comportamental possui uma dependência majoritária do hipocampo, entretanto os córtex parietal e pré-frontal também são importantes estruturas relacionadas à memória espacial (Morris, 1984; D'Hooge e De Deyn, 2001; Saito e Watanabe, 2006; Save e Poucet, 2009). Streck e cols. (2002a; 2003a; 2003b) mostraram que a Hcy modifica uma série de parâmetros oxidativos, e de metabolismo energético em hipocampo de ratos que podem estar associados às alterações observadas na memória. O córtex pré-frontal também parece ser afetado pela administração crônica de Hcy (Matté et al., 2004). A manutenção da integridade da Na^+,K^+-ATPase é de fundamental importância para o aprendizado espacial (Zhan et al., 2004; Moseley et al., 2007) e a LTP (Glushchenko e Izvarina, 1997). Nesse contexto, Moseley e cols. (2007) mostraram que camundongos heterozigotos para a ausência das subunidades $\alpha 2$ ou $\alpha 3$ possuem um pronunciado déficit no aprendizado espacial, medido na tarefa do labirinto aquático de Morris.

No presente estudo mostramos que a hiper-homocisteinemia crônica inibe a atividade da Na^+,K^+-ATPase em córtex parietal de ratos sacrificados 12 horas após a última administração de Hcy, retornando a níveis de controle após 31 dias. A redução na atividade dessa enzima de membrana possivelmente ocorre via dano oxidativo, considerando a ampla literatura mostrando que essa enzima é sensível ao estresse oxidativo, em especial à peroxidação lipídica (Dobrota et al., 1999; Rauchová, Drahota e Koudelová, 1999; Wyse et al., 2002; Sridevi et al., 2007), que também foi demonstrada nesse estudo (Matté et al., 2007). Nesse contexto, a presente tese descreveu o efeito pró-oxidante da Hcy, que foi evidenciado através da redução das defesas antioxidantes, bem como o resultante dano ao DNA (Matté et al., 2009a) e peroxidação lipídica em córtex parietal de ratos (Matté et al., 2007). Corroborando com nossos dados, Lima e cols. (2008) correlacionam a indução de peroxidação lipídica e protéica, assim como a redução na atividade da Na^+,K^+-ATPase em córtex parietal, com o déficit no aprendizado espacial em ratos submetidos ao modelo de injúria traumática cerebral. Wyse e cols. (2002) demonstraram que a inibição da atividade da Na^+,K^+-ATPase causada pela administração aguda de Hcy é prevenida pelo tratamento com as vitaminas E & C, reforçando a participação do estresse oxidativo na alteração da atividade dessa enzima. Entretanto não podemos descartar um possível efeito da Hcy sobre a síntese ou degradação dessa enzima, o que pode afetar a atividade específica, que foi medida nesse estudo, através da redução no conteúdo total enzimático (Iannello, Milazzo e Belfiore, 2007). Acreditamos que as alterações na atividade da Na^+,K^+-ATPase verificadas em hipocampo, córtex parietal e pré-frontal induzidas pela Hcy (Streck et al., 2002a; Matté et al., 2004; Matté et al., 2006) possam estar associadas ao déficit de memória espacial verificado nessa tese.

A fim de avaliar se o ácido fólico é capaz de prevenir as alterações induzidas pela Hcy, nós realizamos o tratamento concomitante com essa vitamina do 6º ao 28º dia de vida dos ratos. O ácido fólico preveniu o aumento na latência para cruzar o local da plataforma apresentado pelos ratos tratados com Hcy, entretanto não foi capaz de prevenir a redução no número de cruzamentos sobre o alvo na sessão de teste da memória espacial de referência. O prejuízo na memória de trabalho, demonstrado pelos ratos hiper-homocisteinêmicos, foi prevenido pela administração concomitante de ácido fólico. Apesar do ácido fólico estar sendo utilizado em uma série de estudos clínicos a fim de prevenir déficits cognitivos (Kado et al., 2005; Durga et al., 2007; Malouf e Grimley Evans, 2008), poucos trabalhos em animais tem avaliado o mecanismo de ação dessa vitamina, frequentemente concentrando-se na relação metabólica entre o ácido fólico e a Hcy (Huang et al., 2007; Zhang et al., 2009). Nossos resultados mostram que o tratamento com ácido fólico apresentou um comportamento antioxidante, reduzindo significativamente o TBARS, um índice de peroxidação lipídica, assim como prevenindo o aumento do TBARS e a inibição da Na^+,K^+-ATPase em córtex parietal de ratos tratados cronicamente com Hcy. Apesar do ácido fólico participar do metabolismo da Hcy, através da doação de grupos metil via N^5-metiltetraidrofolato (Fowler, 1997; Brosnan et al., 2004; Finkelstein, 2007), o tratamento com ácido fólico não foi capaz de reduzir os níveis plasmáticos e cerebrais de Hcy em ratos tratados cronicamente com esse aminoácido, em nosso modelo experimental. Existem indícios de que os efeitos benéficos do ácido fólico sobre as alterações comportamentais e bioquímicas induzidas pela Hcy sejam independentes da sua participação no catabolismo desse aminoácido, parecendo estar mais relacionado à sua atividade antioxidante (Joshi et al., 2001; Au-Yeung et al., 2006; Patro et al., 2006). Nesse sentido, o capítulo I da presente tese traz resultados que reforçam as propriedades antioxidantes do ácido fólico, desde que a

administração crônica dessa vitamina preveniu a redução nas defesas antioxidantes, bem como o dano ao DNA em córtex parietal de ratos hiper-homocisteinêmicos (Matté et al., 2009a).

Nosso próximo objetivo consistiu na avaliação do efeito dessa vitamina do complexo B sobre o déficit na memória aversiva, provocada pela administração aguda de Hcy imediatamente após a sessão de treino na tarefa de esquiva inibitória. Dados prévios do nosso laboratório mostram que uma única administração de Hcy prejudicou a aquisição, a consolidação e a evocação da LTM; e que o pré-tratamento com a combinação antioxidante de vitaminas E & C preveniu tal efeito, sugerindo um possível mecanismo de ação da Hcy (Reis et al., 2002). Nossos resultados confirmaram os dados de Reis e cols. (2002), mostrando que a administração aguda de Hcy promoveu um déficit na consolidação das memórias de curta e longa duração, quando os animais foram testados 1 e 24 horas após o treino, respectivamente. O pré-tratamento com ácido fólico preveniu completamente o prejuízo na consolidação da memória provocado pela Hcy. Também mostramos que a administração de uma única dose de ácido fólico promove um incremento nos níveis plasmáticos de folato, atingindo cerca de 13,5 µg/mL, 15 minutos após a administração, e retornando a níveis de controle em aproximadamente 3 horas. O teste de campo aberto foi utilizado como controle experimental, a fim de descartar alterações motoras causadas pelos tratamentos.

O BDNF tem sido reportado como uma neurotrofina indispensável à consolidação da memória, tanto da STM quanto da LTM, sendo encontrado em altas concentrações no hipocampo e no córtex, onde exerce seus efeitos em neurônios ativados via receptores TrkB (Alonso et al., 2002; Alonso et al., 2005; Bramham e Messaoudi, 2005; Soulé, Messaoudi e Bramham, 2006; Bekinschtein et al., 2008). Nossos dados mostram que a administração de Hcy, imediatamente após o estímulo

para o aprendizado no aparato de esquiva inibitória, reduz os níveis de BDNF em hipocampo de ratos sacrificados 1 hora após a injeção de Hcy. Vinte e quatro horas após, os níveis de BDNF hipocampais eram comparáveis aos controles. Por outro lado, o imunoconteúdo de BDNF no córtex parietal não foi afetado em nenhum dos tempos avaliados (1 e 24 horas após o estímulo para o aprendizado). A administração prévia de ácido fólico foi capaz de prevenir a redução de BDNF hipocampal observada nos ratos tratados agudamente com Hcy.

Alonso e cols. (2002) mostraram que o BDNF hipocampal endógeno é necessário para a consolidação da memória, desde que a infusão de anticorpo anti-BDNF 1 hora após o treino no aparato de esquiva inibitória, prejudica a LTM. Por outro lado, a STM foi alterada somente quando o anti-BDNF foi infundido antes do treino, não sendo efetivo quando injetado após o estímulo para o aprendizado. Considerando esses dados, acreditamos que a redução nos níveis hipocampais de BDNF esteja associada ao déficit na consolidação da LTM, induzido pela Hcy.

A redução do conteúdo de BDNF pode estar associada ao estresse oxidativo e a neurotoxicidade glutamatérgica. Zou e Crews (2006) mostraram que o estado redox oxidante e a excitotoxicidade via ativação de receptores NMDA reduziram a expressão do mRNA do BDNF em culturas organotípicas de hipocampo e córtex entorrinal, assim como a ligação de CREB ao DNA, que é responsável pelo aumento da expressão gênica, inclusive do próprio BDNF. Em concordância, Pugazhenthi e cols. (2006) avaliaram o efeito do estresse oxidativo sobre a expressão de BDNF em neurônios hipocampais, e demonstraram que produtos da peroxidação lipídica (acroleína e HNE) reduziram a atividade promotora do gene do BDNF, sugerindo uma redução na atividade do CREB. Existem evidências claras de que a Hcy induz estresse oxidativo, via ativação de receptores glutamatérgicos (Kim e Pae, 1996; Ho et al., 2003; Jara-

Prado et al., 2003) e via auto-oxidação (Mudd, Levy e Kraus, 2001; Dayal et al., 2004; Faraci e Lentz, 2004). Além disso, dados do nosso grupo de pesquisa mostram que a Hcy *in vitro* promove a peroxidação lipídica e reduz as defesas antioxidantes em hipocampo de ratos (Streck et al., 2003b), o que pode contribuir para a redução nos níveis dessa neurotrofina, e consequentemente resultar no prejuízo cognitivo observado em nosso estudo.

Esses dados corroboram com os resultados apresentados no capítulo V (Matté et al., 2007), desde que a memória espacial também parece ser dependente do BDNF hipocampal. Nesse contexto, Heldt e cols. (2007) mostraram que a redução nos níveis de BDNF hipocampais, via deleção do gene do BDNF em camundongos adultos, resulta em um déficit mnemônico no teste do labirinto aquático de Morris. Além disso, camundongos geneticamente modificados, superexpressando BDNF em córtex e hipocampo, apresentam melhor performance no teste do labirinto aquático de Morris, quando comparados a camundongos selvagens (Nakajo et al., 2008).

Os mecanismos que afetam a STM e a LTM ainda não estão completamente elucidados, e certamente não dependem de apenas um único fator, tal qual o BDNF, portanto mais estudos são necessários para esclarecer os complexos fatores relacionados ao mecanismo de ação da Hcy sobre os processos de aprendizado e memória. Nesse contexto, discutimos acima o efeito da hiper-homocisteinemia crônica sobre a atividade da Na^+,K^+-ATPase, e vimos que a Hcy inibe a atividade dessa enzima em córtex parietal (Matté et al., 2004; 2006; 2007) e hipocampo (Streck et al., 2002a; 2002b; Wyse et al., 2002). A memória aversiva é dependente da atividade dessa enzima, como demonstrado por Sato e cols. (2004), que, através da administração de ouabaína, inibiram a atividade Na^+,K^+-ATPase em camundongos e observaram um prejuízo no aprendizado na tarefa de esquiva inibitória. Podemos aventar ainda outras hipóteses, que podem ser

complementares ao efeito oxidativo da Hcy, sobre o prejuízo na memória determinado por essa neurotoxina. A disfunção mitocondrial induzida pela Hcy nessa tese e em estudos anteriores (Streck et al., 2003a), através de alterações nos complexos da cadeia respiratória e no ciclo de Krebs, podem resultar na geração de ERO, bem como na depleção de ATP (Kowaltowski et al., 2009). A excitotoxicidade glutamatérgica, resultado da inibição da captação de glutamato pela Hcy, pode estar relacionada com a disfunção mitocondrial, o estresse oxidativo e, em última instância à morte celular, caracterizando a neurodegeneração observada em alguns estudos (Kruman et al., 2000; Ho et al., 2002; Zieminska e Lazarewicz, 2006), e que podem estar associados aos déficits cognitivos apresentados pelos animais tratados com Hcy.

No capítulo que trata sobre a memória espacial já havíamos mostrado o efeito neuroprotetor do ácido fólico, prevenindo o prejuízo na memória, assim como a inibição da Na^+,K^+-ATPase em córtex parietal de ratos submetidos à hiper-homocisteinemia crônica (Matté et al., 2007). Nesse capítulo mostramos que o pré-tratamento com ácido fólico preveniu os efeitos da administração aguda de Hcy sobre a consolidação da memória de curta e longa duração, e sobre os níveis de BDNF em hipocampo.

O déficit na memória induzido pela administração de Hcy tem um mecanismo complexo, multifatorial e inter-relacionado, que possivelmente envolve o estresse oxidativo, a disfunção mitocondrial, a excitotoxicidade glutamatérgica, a inibição da atividade da Na^+,K^+-ATPase, bem como a redução de neurotrofinas, como o BDNF. A administração de ácido fólico preveniu os efeitos neurotóxicos bioquímicos e comportamentais induzidos pela administração de Hcy, provavelmente envolvendo um mecanismo antioxidante.

V. CONCLUSÕES

Os resultados da presente tese permitem concluir que:

1. A administração de ácido fólico preveniu o estresse oxidativo observado em córtex parietal de ratos submetidos ao tratamento crônico com Hcy, que apresentaram redução no conteúdo de antioxidantes não-enzimáticos e enzimáticos (CAT e GPx), bem como peroxidação lipídica e dano ao DNA.

2. O tratamento concomitante com ácido fólico também foi capaz de prevenir os danos oxidativos observados perifericamente em ratos submetidos à administração crônica de Hcy, tais como aumento na atividade das enzimas antioxidantes SOD e CAT em eritrócitos, redução das defesas antioxidantes não-enzimáticas em plasma, e dano ao DNA nas células nucleadas periféricas.

3. A administração crônica de Hcy promoveu o estresse oxidativo em tecido hepático de ratos, demonstrado pela redução nas defesas antioxidantes enzimáticas (CAT) e não-enzimáticas, assim como pelo aumento da peroxidação lipídica. A redução no conteúdo de grupos tióis também foi observada e pode estar relacionada com a redução das defesas antioxidantes, tais como a GSH, e/ou com a oxidação de proteínas hepáticas.

4. Estudos histológicos mostraram que a administração crônica de Hcy não promove alterações morfológicas significativas no tecido hepático de ratos, entretanto infiltrado inflamatório foi observado, bem como um aumento na fibrose extracelular e a redução no conteúdo de glicogênio/glicoproteínas. Esses efeitos possivelmente estão associados ao estresse oxidativo promovido pela Hcy e à ativação de células estreladas hepáticas, responsáveis pela produção e acúmulo de matriz extracelular.

5. As administrações aguda e crônica de Hcy não alteraram a atividade da CK, da SDH e do complexo II da cadeia respiratória em córtex parietal de ratos. Animais submetidos a uma única injeção de Hcy apresentaram inibição na atividade da COX, enquanto ratos cronicamente tratados com Hcy apresentaram elevação na atividade dessa enzima. O tratamento com ácido fólico preveniu tais efeitos, provavelmente atuando como antioxidante.

6. As administrações aguda e crônica de Hcy inibem a captação de glutamato em córtex parietal de ratos em diferentes estágios de desenvolvimento do SNC, em um mecanismo associado à redução do imunoconteúdo de GLAST e GLT-1, ao menos na hiper-homocisteinemia crônica.

7. O tratamento concomitante com ácido fólico preveniu o déficit cognitivo espacial apresentado por ratos adultos tratados cronicamente com Hcy, na tarefa do labirinto aquático de Morris. A ação do ácido fólico parece estar associada à sua atividade antioxidante, bem como à prevenção da inibição da Na^+,K^+-ATPase em córtex parietal, sendo independente do seu efeito sobre o metabolismo da Hcy.

8. O pré-tratamento com ácido fólico preveniu o déficit na consolidação das memórias aversivas de curta e longa duração, avaliadas na tarefa de esquiva inibitória, em ratos submetidos à administração aguda de Hcy. A redução nos níveis hipocampais de BDNF em ratos hiper-homocisteinêmicos também foi prevenida pela administração de ácido fólico, enquanto o imunoconteúdo de BDNF não foi alterado em córtex parietal.

Essa tese mostrou os efeitos neurotóxicos da Hcy, via indução de estresse oxidativo, promovendo disfunção mitocondrial e glutamatérgica, que possivelmente estão refletidos no prejuízo das memórias espacial e aversiva exibidas pelos ratos hiper-homocisteinêmicos. A injúria mediada por esse aminoácido não se restringe ao SNC,

desde que observamos dano oxidativo em sangue e fígado de ratos tratados com Hcy. Nossa hipótese inicial de que o ácido fólico poderia prevenir os danos causados pela administração de Hcy se confirmou, o que nos permite sugerir a avaliação dessa vitamina antioxidante como uma terapia adjuvante em doenças cuja fisiopatologia esteja associada à hiper-homocisteinemia.

VI. PERSPECTIVAS

A presente tese responde algumas questões referentes aos efeitos da Hcy e do ácido fólico sobre parâmetros de estresse oxidativo, metabolismo energético, captação de glutamato, bem como sobre a memória em ratos Wistar. Entretanto, diversos aspectos permanecem por ser esclarecidos. Nesse sentido, o presente estudo amplia as possibilidades de trabalho em diversas áreas do conhecimento bioquímico e comportamental, e deixa muitas perspectivas de estudo.

a) Verificar o efeito da administração de Hcy sobre:

1. a expressão das enzimas antioxidantes em tecidos do SNC e periféricos;
2. as células estreladas hepáticas, sobre o conteúdo de matriz extracelular, bem como sobre a síntese, degradação e conteúdo de glicogênio em fígado de ratos;
3. outros parâmetros do metabolismo energético em córtex parietal de ratos, tais como a captação de glicose, a produção de lactato, a produção de CO_2, e a atividade de outras enzimas do ciclo de Krebs e da cadeia transportadora de elétrons;
4. parâmetros de dano mitocondrial em córtex parietal de ratos, tais como a liberação de citocromo *c*, o inchamento mitocondrial, e o envolvimento do Ca^{2+} nos efeitos mediados pela Hcy;
5. outros parâmetros relacionados ao sistema glutamatérgico, tais como a liberação e o ligação de glutamato em córtex parietal de ratos;
6. a morte celular em estruturas do SNC, caracterizando o tipo de morte e os mecanismos envolvidos.

b) Investigar o efeito do ácido fólico e de outros compostos antioxidantes sobre as alterações bioquímicas e comportamentais causadas pela Hcy, no intuito de fortalecer os

dados de pesquisa básica, a fim de permitir a utilização desses resultados como contexto inicial para o estudo desses antioxidantes em pacientes homocistinúricos ou comprometidos por outras doenças associadas à hiper-homocisteinemia.

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