Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

Avaliação de parâmetros inflamatórios e de estresse oxidativo em modelo experimental de hiper-homocisteinemia severa

Aline Andrea da Cunha

Orientadora: Prof.ª Dr.ª Angela Terezinha de Souza Wyse

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Dedico essa tese aos meus pais, exemplos de amor, dedicação, perseverança e cumplicidade com os filhos. Nos quais procuro me espelhar em todos os momentos da minha vida!

Ciência

"Se o plantio é necessário,
suficiente não será
para que a planta da ciência
viceje ao longo da vida.

Não se pode confiar
nas volúveis nuvens
do saber definitivo
nem nos imprevisíveis
ventos das novidades.

Na vigília permanente,
é preciso manter sempre
firmes as raízes da perseverança,
flexíveis os caules da imaginação,
verdes as folhas da paixão."

(Clóvis Wannmacher)

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SUMÁRIO

RESUMO

A homocistinúria clássica 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, déficit cognitivo, crises convulsivas, alterações vasculares, esqueléticas, oculares, hepáticas, e pulmonares, cuja fisiopatologia não está totalmente elucidada. O objetivo geral da presente tese foi avaliar o efeito da hiper-homocisteinemia severa sobre parâmetros inflamatórios (citocinas, quimiocinas, óxido nítrico, proteínas de fase aguda e contagem diferencial de leucócitos) e outros parâmetros relacionados, tais como o sistema da coagulação, o estresse oxidativo e, as vias de sinalização da Akt e GSK-3β no cérebro e/ou sangue de ratos. Além disso, investigamos o efeito do estresse oxidativo em pulmão de ratos submetidos à hiper-homocisteinemia severa. A hiper-homocisteinemia aguda aumentou as citocinas pró-inflamatórias (TNF-α, IL-1β e IL-6), a quimiocina CCL_2 (MCP-1) e os níveis de nitritos no hipocampo e no córtex cerebral de ratos. Os níveis de TNF-α e IL-6 também aumentaram no soro de ratos submetidos à administração aguda de homocísteina. A percentagem de neutrófilos e monócitos aumentou no sangue após a administração aguda de homocisteína; entretanto, não observamos alterações nos níveis das proteínas de fase aguda (PCR e α_1-glicoproteína ácida). A hiper-homocisteinemia crônica severa também aumentou as citocinas pró-inflamatórias (TNF-α, IL-1β e IL-6), a quimiocina CCL_2 (MCP-1) e os níveis de prostaglandina E_2 no hipocampo e/ou soro de ratos. Os níveis de nitritos aumentaram no hipocampo, mas verificamos uma diminuição em seus níveis no soro após a hiper-homocisteinemia crônica. O imunoconteúdo citoplasmático e nuclear da subunidade p65 do NF-κB e a atividade da acetilcolinesterase aumentaram no hipocampo de ratos submetidos à hiper-homocisteinemia crônica. Demonstramos também que a hiper-homocisteinemia aguda severa aumentou a contagem de plaquetas no sangue e os níveis de fibrinogênio no plasma. O tempo de protrombina, tempo de tromboplastina parcial ativada e os níveis de nitritos no plasma diminuíram após a administração aguda de homocisteína. Mostramos que a hiper-homocisteinemia aguda severa aumentou as espécies reativas ao ácido tiobarbitúrico, um índice de peroxidação lipídica e, diminuiu a atividade das enzimas antioxidantes, superóxido dismutase e glutationa peroxidase e aumentou a atividade da enzima catalase em eritrócitos de ratos. Em relação ao envolvimento das vias de sinalização, demonstramos que a hiper-homocisteinemia aguda severa aumentou a fosforilação da Akt, o imunoconteúdo citoplasmático e nuclear da subunidade p65 do NF-κB e a fosforilação da proteína *Tau*, mas reduziu a fosforilação da GSK-3β após a administração aguda de homocisteína. Por outro lado, a hiper-homocisteinemia crônica severa não alterou a fosforilação da Akt e GSK-3β após a administração crônica desse aminoácido. Além disso, demonstramos que a hiper-homocisteinemia crônica severa aumentou a peroxidação lipídica e, reduziu as defesas antioxidantes enzimáticas e não-enzimáticas em pulmão de ratos. Em resumo, nossos resultados demonstram que a inflamação, a hipercoagulabilidade e o estresse oxidativo podem ocorrer após a hiper-homocisteinemia severa, o que pode estar associado, pelo menos em parte, com as alterações cerebrais e cardiovasculares, bem como no tromboembolismo pulmonar que estão presentes em alguns pacientes homocistinúricos.

ABSTRACT

Classical homocystinuria, an inborn error of metabolism, is biochemically characterized by cystathionine β-synthase deficiency and homocysteine tissue accumulation. Mental retardation, cognitive deficiency, seizures, vascular, skeletal, ocular, hepatic and pulmonary complications are common symptoms in homocystinuric patients. However, the mechanisms underlying these alterations are not fully understood. The main objective of this doctoral thesis was to investigate the effect of severe hyperhomocysteinemia on inflammatory parameters (cytokines, chemokines, nitric oxide, acute phase-proteins and a differential count of leukocytes) and other related parameters, such as the coagulation system, oxidative stress as well as the involvement of the Akt and GSK-3β pathway in the brain and/or blood of rats. In addition, we investigated the effect of oxidative stress in the lungs of rats submitted to severe hyperhomocysteinemia. Acute hyperhomocysteinemia increased pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), chemokine CCL_2 (MCP-1) and nitrite levels in hippocampus and cerebral cortex of rats. TNF-α and IL-6 levels also increased in serum of rats subjected to acute homocysteine administration. The percentage of neutrophils and monocytes increased in blood after acute homocysteine administration, but acute phase-proteins (CRP and α_1-acid glycoprotein) were not altered by homocysteine administration. In addition, we showed that chronic hyperhomocysteinemia increased pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), chemokine CCL_2 (MCP-1) and prostaglandin E_2 levels in the hippocampus and/or serum of rats. Nitrite levels increased in the hippocampus, but decreased in the serum after chronic homocysteine administration. The immunocontent of cytosolic and nuclear NF-κB/p65 subunit and the acetylcholinesterase activity were increased in the hippocampus of rats subjected to chronic hyperhomocysteinemia. Next, we demonstrated that acute hyperhomocysteinemia increased the platelet counts in the blood and plasma fibrinogen levels of rats. Prothrombin time, activated partial thromboplastin time, and nitrite levels decreased in plasma after acute hyperhomocysteinemia. Additionally, we showed that acute hyperhomocysteinemia increased thiobarbituric acid-reactive, an index of lipid peroxidation, and decreased the superoxide dismutase and gluthatione peroxidase activity and also increased the catalase activity in erythrocytes of rats. Moreover, acute hyperhomocysteinemia increases Akt phosphorylation, cytosolic and nuclear immunocontent of NF-κβ/p65 subunit and *Tau* protein phosphorylation, but reduces GSK-3β phosphorylation after acute homocysteine administration. On the other hand, chronic hyperhomocysteinemia did not alter Akt and GSK-3β phosphorylation after the chronic administration of this amino acid. We also showed that chronic hyperhomocysteinemia increased lipid peroxidation, and disrupted antioxidant defenses (enzymatic and non-enzymatic) in the lungs of rats. In summary, our findings showed that inflammation, hypercoagulability and oxidative stress can occur after severe hyperhomocysteinemia, possibly in association, at least in part, with cerebral and cardiovascular dysfunction, as well as pulmonary thromboembolism complications observed in some homocystinuric patients.

LISTA DE ABREVIATURAS

AA: ácido araquidônico
ACh: acetilcolina
AChE: acetilcolinesterase
ADP: difosfato de adenosina
AKT: proteína cinase B
ATP: trifosfato de adenosina
BDNF: fator neurotrófico derivado do encéfalo e cérebro
BHMT: betaína homocisteína metiltransferase
CAT: catalase
CBS: cistationina β-sintase
CD11b: integrina $\alpha_M\beta_2$
CGL: cistationina γ-liase
cNOS: óxido nítrico sintase constitutiva
COX-2: ciclooxigenase-2
DNA: ácido desoxirribonucleico
EIM: erros inatos do metabolismo
eNOS: óxido nítrico sintase endotelial
ERNS: espécies reativas de nitrogênio
EROS: espécies reativas de oxigênio
ERK: cinase regulada por sinais extracelulares
GLAST- transportador glial de glutamato
GLT-1- transportador glutamato-aspartato dependente de Na^+
GSH: glutationa (forma reduzida)
GSK-3β: glicogênio sintase cinase-3 beta
GSSG: glutationa (forma oxidada)
GPx: glutationa peroxidase
H_2O_2: peróxido de hidrogênio
HCU: homocistinúria
ICAM-1: molécula de adesão intercelular
Iκβ: subunidade inibitória do fator de transcrição nuclear-kappaB
IL-1β: interleucina-1β

IL-6: interleucina 6

iNOS: óxido nítrico sintase induzível

JNK: proteína cinase de C-Jun N-terminal

KTTP: tempo de tromboplastina parcial ativada

LPS: lipopolissacarídeo

MAPK: proteína cinase ativada por mitógenos

MAT: adenosiltransferase

MCP-1: proteína quimiotática de monócito do tipo 1

5-MeTHF: 5-metiltetraidrofolato

MS: metionina sintase

NADP: nicotinamida adenina dinucleotídeo fosfato

NADPH: nicotinamida adenina dinucleotídeo fosfato reduzida

NADPH oxidase: nicotinamida adenina dinucleotídeo fosfato oxidase

NF-kB: fator de transcrição nuclear-kappaB

NMDA: N-metil-D-aspartato

NO: óxido nítrico

NOS: óxido nítrico sintase

nNOS: óxido nítrico sintase neuronal

O_2: oxigênio molecular

$^1O_{2:}$ oxigênio singlet

O_2^-: ânion superóxido

OH^-: ânion hidroxila

$ONOO^-$: ânion peroxinitrito

PCR: proteína C reativa

PDK-1: cinase dependente de fosfoinositois-1

PDK-2: cinase dependente de fosfoinositois-2

PI3K: fosfatidilinositol-3-cinase

PKC: proteína cinase C

PKA: proteína cinase A

PLP: piridoxal fosfato

SAM: S-adenosilmetionina

SAH: S-adenosil homocisteína

SAHH: S-adenosil homocisteína hidrolase

SNC: sistema nervoso central

SOD: superóxido dismutase

TBARS: substâncias reativas ao ácido tiobarbitúrico

TGF-β: fator de crescimento tumoral beta

TNF-α: fator de necrose tumoral alfa

TP: tempo de protrombina

TRAP: potencial antioxidante total

VCAM-1: molécula de adesão vascular

PARTE I

1. INTRODUÇÃO

1.1. Erros Inatos do Metabolismo

Os erros inatos do metabolismo (EIM) são, majoritariamente, doenças hereditárias, multifatoriais, majoritariamente, autossômicas recessivas que se caracterizam pela síntese de uma proteína alterada, geralmente uma enzima com atividade parcial ou totalmente reduzida, resultando no bloqueio da via metabólica com consequente acúmulo de substrato e diminuição na síntese de produto. O bloqueio metabólico pode, ainda, originar outras substâncias tóxicas por rotas metabólicas alternativas (Mudd et al., 2001).

Aproximadamente 500 EIM diferentes foram identificados até o momento, correspondendo a cerca de 10% de todas as doenças genéticas (Mudd et al., 2001). Individualmente, as doenças decorrentes dos EIM são consideradas raras. Porém, em conjunto, apresentam alta frequência, acometendo aproximadamente 1:1000 recém-nascidos vivos (Giugliani e Coelho, 1997).

A classificação mais utilizada para os EIM é realizada de acordo com a área do metabolismo afetada: EIM de aminoácidos, de ácidos orgânicos, de glicídios, de lipídios, de glicosaminoglicanos, de glicoproteínas, de purinas e pirimidinas, de enzimas eritrocitárias, de metais, de lipoproteínas, de hormônios e de proteínas plasmáticas. Dentre os mais frequentes, estão os EIM de aminoácidos que incluem a Homocistinúria Clássica (HCU), objeto de estudo desta tese.

1.2. Homocisteína

A homocisteína (Hcy) é um aminoácido sulfurado não-protéico derivado da metionina (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 adenosiltransferase (MAT, EC 2.5.1.6), recebendo um grupo adenosil do trifosfato de adenosina (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 cistationina β-sintase (CBS; EC 4.2.1.22), 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; Stead et al., 2004; Finkelstein, 2007).

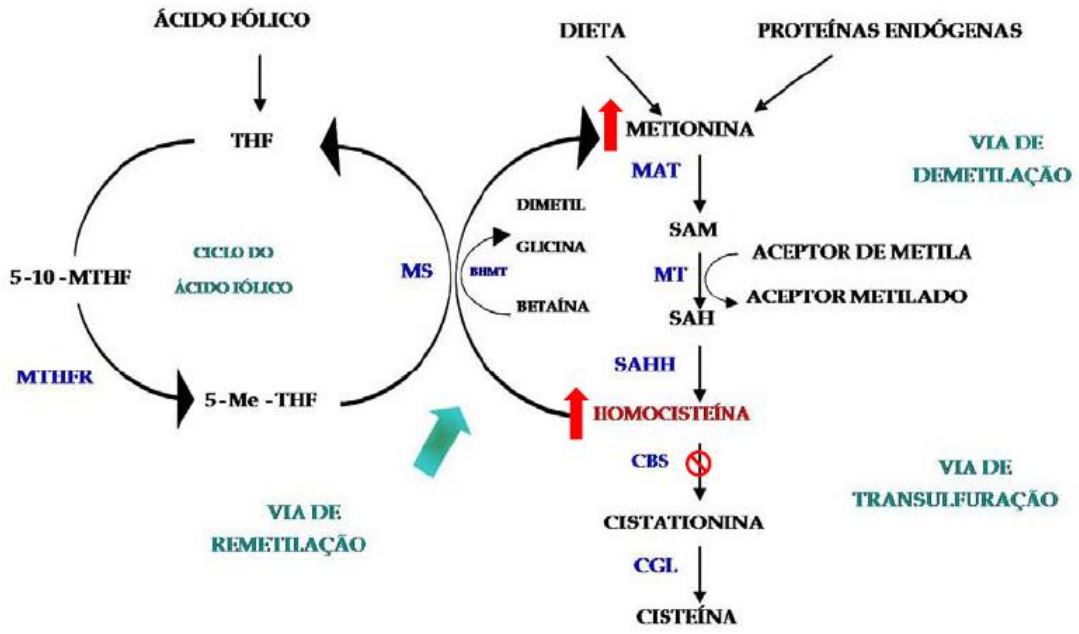

Figura 1: Metabolismo da homocisteína. MAT - metionina adenosil transferase; MT - metiltransferase; SAHH - S-adenosilhomocisteína hidrolase; CBS - cistationina β-sintase; CGL - cistationina γ-liase; MS - metionina sintase; BHMT - betaína homocisteína metiltransferase; MTHFR - metilenotetrahidrofolato redutase; SAM - S-adenosil metionina; SAH - S-adenosil homocisteína; THF - tetrahidrofolato; 5,10-MTHF - 5,10-metilenotetrahidrofolato; 5-Me-THF - 5-metiltetrahidrofolato (Adaptado de Mudd et al., 2001).

1.3. Homocistinúria Clássica

A HCU clássica é um EIM dos aminoácidos sulfurados, associado à deficiência da enzima CBS. 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 clássica apresenta uma prevalência mundial média variando de 1:200.000 à 1:335.000 nascidos vivos (Mudd et al., 2001). Entretanto, no Catar, essa prevalência alcança 1:1800, devido à organização tribal dessa população, onde ocorrem muitos casamentos consanguíneos que propagam uma mutação na enzima CBS (Zschocke et al., 2009).

Bioquimicamente, a HCU clássica é caracterizada pelo acúmulo tecidual de Hcy, acompanhado pela elevação dos níveis de seu precursor, o aminoácido essencial Met, assim como a 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, característica que resultou na denominação da doença. As concentrações plasmáticas normais de Hcy variam de 5-15 μM, podendo atingir 500 μM em pacientes homocistinúricos não tratados. Pacientes portadores de HCU clássica apresentam uma considerável redução na atividade da enzima CBS em fígado, fibroblastos, cérebro, linfócitos e vilosidades coriônicas (Mudd et al., 2001).

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

às altas concentrações teciduais de Hcy observadas nos pacientes (Mudd et al., 2001; Jiang et al., 2005).

O diagnóstico da HCU clássica é dado através da combinação de sinais clínicos e das avaliações bioquímica e molecular. 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 (Fowler e Jakobs, 1998; Mudd et al., 2001).

Após o diagnóstico clínico e bioquímico da HCU, inicia-se um tratamento com uma dieta reduzida em Met e com uma suplementação da vitamina B_6, cisteína e ácido fólico. 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. A piridoxina (vit B_6) é convertida a PLP, co-fator da CBS, e, portanto estimula a atividade dessa enzima reduzindo a concentração de Hcy. O ácido fólico é convertido a 5-metiltetraidrofolato (5-MeTHF), um doador de grupamentos metil na conversão de Hcy à Met (Dias et al., 2001 Gebara e Matioli, 2006). 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 et al., 2001).

1.4. Aspectos tóxicos da Homocisteína

Embora a hiper-homocisteinemia seja um achado bioquímico característico da HCU clássica (Mudd et al., 2001), essa condição metabólica, mesmo em baixas concentrações, também tem sido demonstrada em pacientes acometidos de doenças neurodegenerativas (Diaz-Arrastia, 2000), psiquiátricas (Sachdev, 2004; Bottiglieri, 2005), vasculares (Clarke et al., 1991; Welch e Loscalzo, 1998; Faraci e Lentz, 2004), hepáticas (Adinolfi et al., 2005) e pulmonares (Jiang et al., 2005; Hamelet et al., 2007). 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.

O cérebro é um órgão especialmente sensível aos efeitos da Hcy; sua neurotoxicidade se deve principalmente a sua capacidade de ser captado através de um transportador específico de membrana (Grieve et al., 1992). Outro fator importante que favorece a neurotoxicidade da Hcy é atribuído à ausência de duas vias importantes de eliminação dessa substância, já que a enzima BHMT e parte da via de transulfuração até cisteína não estão presentes no cérebro (Finkelstein, 2007). Nesse contexto, a Hcy tem sido relacionada à morte neuronal via excitotoxicidade, por provocar a ativação de receptores glutamatérgicos metabotrópicos do grupo I (Ziemińska et al., 2003) e ionotrópico N-metil-*D*-aspartato (NMDA) (Kim e Pae, 1996; Lipton et al., 1997). Trabalho realizado pelo nosso grupo de pesquisa demonstrou, recentemente, que a Hcy reduz a captação de glutamato e o imunoconteúdo dos transportadores gliais, GLAST e GLT-1, em córtex parietal de ratos tratados cronicamente com Hcy (Matté et al., 2010). Além disso, Ho e cols. (2002) demonstraram que a Hcy promove o acúmulo citosólico de Ca^{+2}, hiperfosforilação da proteína *Tau*, além de induzir apoptose e estresse oxidativo.

Outra abordagem utilizada para explicar os efeitos citotóxicos da Hcy envolve a indução de estresse oxidativo, via aumento na produção de espécies reativas de oxigênio (EROS), que podem atuar sobre lipídeos, proteínas e sobre o DNA ou, por redução na produção de defesas antioxidantes (Halliwell e Whiteman, 2004). Estudos têm demonstrado que a auto-oxidação da Hcy formando homocistina e dissulfetos mistos promove a geração de EROS, como o radical superóxido ($O_2^{\cdot -}$) e peróxido de hidrogênio (H_2O_2), que posteriormente é convertido a radical hidroxil (OH^-), o mais danoso dentre as EROS (Dayal et

al., 2004; Faraci e Lentz, 2004). Resultados obtidos em nosso laboratório corroboram com dados da literatura, os quais mostram que a Hcy promove peroxidação lipídica e aumenta a produção de EROS, além de diminuir as defesas antioxidantes enzimáticas e os níveis de nitritos em coração de ratos (Kolling et al., 2011). Além disso, também demonstramos que a Hcy induz estresse oxidativo no hipocampo, córtex parietal e fígado de ratos (Streck et al., 2003a; Matté et al., 2009a; Matté et al., 2009b).

O sistema vascular também é bastante comprometido nos pacientes com HCU clássica. O tromboembolismo é a maior causa de mortalidade entre esses pacientes. Dentre os eventos resultantes do tromboembolismo, pode-se citar a oclusão de veias e artérias periféricas, que pode resultar em embolismo pulmonar, acidentes vasculares cerebrais e infarto do miocárdio (McCully, 1996; Mudd et al., 2001; Faraci e Lentz, 2004). A Hcy parece alterar as propriedades anticoagulantes das células endoteliais para um fenótipo pró-coagulante (Jacobsen, 1998; den Heijer et al., 1998), além de alterar a morfologia vascular, estimular a inflamação, diminuir a biodisponibilidade do óxido nítrico (NO) e estimular as vias de coagulação (Stanger et al., 2004; Upchurch et al., 1997a). Outros achados demonstram que a Hcy pode promover um estado pró-inflamatório, verificado através de estudos *in vitro* que demonstraram que a Hcy é capaz de induzir um aumento na expressão de várias citocinas pró-inflamatórias em cultura de células endoteliais (Sung et al., 2001; Poddar et al., 2001).

1.5. Modelo experimental de hiper-homocisteinemia severa

Modelos animais são amplamente utilizados para a compreensão da fisiopatologia de doenças humanas. No presente estudo utilizamos um modelo experimental quimicamente induzido de hiper-homocisteinemia severa, a fim de investigar os possíveis mecanismos envolvidos na resposta inflamatória presentes nessa doença, os quais ainda são pouco compreendidos. Nesse modelo, ratos Wistar machos e/ou fêmeas são submetidos a administrações (aguda ou crônica) de Hcy a fim de induzir níveis plasmáticos desse aminoácido semelhantes aqueles encontrados em pacientes com HCU clássica (Mudd et al., 2001; Streck et al., 2002).

1.6. Inflamação

A inflamação pode ser caracterizada como um mecanismo complexo iniciado tanto por fatores endógenos (necrose tecidual), como fatores exógenos (agentes químicos, físicos, estímulo imunológico como, por exemplo, infecção por micro-organismos) (Benjamini et al., 2002; Zhang, 2008). A presença de agentes estranhos ou alguma lesão tecidual serve de estímulo para o início da resposta inflamatória, que tem como objetivo a destruição e a eliminação do agente agressor. O agente invasor é inicialmente reconhecido pelos componentes do sistema imune do organismo, seguido da liberação de mediadores químicos e ativação de diversos tipos de células que se acumulam no sítio da inflamação (Ali et al., 1997; Zhang, 2008).

Inicialmente, durante o processo inflamatório ocorre uma vasoconstrição transitória, seguida de uma vasodilatação induzida pela liberação de

histamina pelos mastócitos ativados. O aumento da permeabilidade vascular leva à formação de edema, quimiotaxia celular e aumento no fluxo sanguíneo. Após esta fase inicial da resposta inflamatória, ocorre uma outra etapa caracterizada por migração de leucócitos e de células fagocíticas para o sítio da lesão. A migração leucocitária, processo no qual as células são atraídas para o tecido lesado, denomina-se de quimiotaxia (Ali et al., 1997; Frangogiannis et al., 2001; Zhang, 2008). Várias células estão envolvidas nesta fase da resposta inflamatória, dentre elas as fagocíticas que são constituídas principalmente de neutrófilos, que fagocitam o agente invasor ou liberam enzimas lisossomais na tentativa de destruir o agente estranho (Benjamini et al., 2002; Zhang, 2008). Caso a inflamação persista, ocorre também, na área lesada, uma infiltração de células do tipo mononucleares, incluindo-se macrófagos e monócitos. Desta forma, com a liberação de mediadores quimiotáticos e a ativação celular, ocorre o que denominamos de amplificação da resposta inflamatória (Benjamini et al., 2002).

Simultaneamente, além da geração de fatores quimiotáticos e uma vasodilatação local, ocorrem também mudanças na distribuição dos componentes celulares em relação ao fluxo sanguíneo. Os leucócitos circulantes, no centro da luz vascular, migram nas proximidades da parede do vaso. Além disso, a migração celular é facilitada pela expressão de moléculas de adesão em diferentes tipos de células (Jung et al., 1998, Siegelman et al., 2000). Os fatores quimiotáticos que estimulam tanto os leucócitos como as células endoteliais a expressarem as moléculas de adesão, também promovem a adesividade entre estas células, processo este conhecido como rolamento. Nesta fase ocorre uma adesão entre os leucócitos e as células

endoteliais (Walzog et al., 1999; Kevil, 2003). A formação de uma interação de alta afinidade entre as células (leucócitos e células endoteliais) com a participação, principalmente das moléculas de adesão do tipo integrinas, permitem uma firme adesão. Assim, os leucócitos então iniciam o processo de transmigração, migrando do vaso para o sítio lesado (Kevil, 2003), conforme podemos observar na Figura 2.

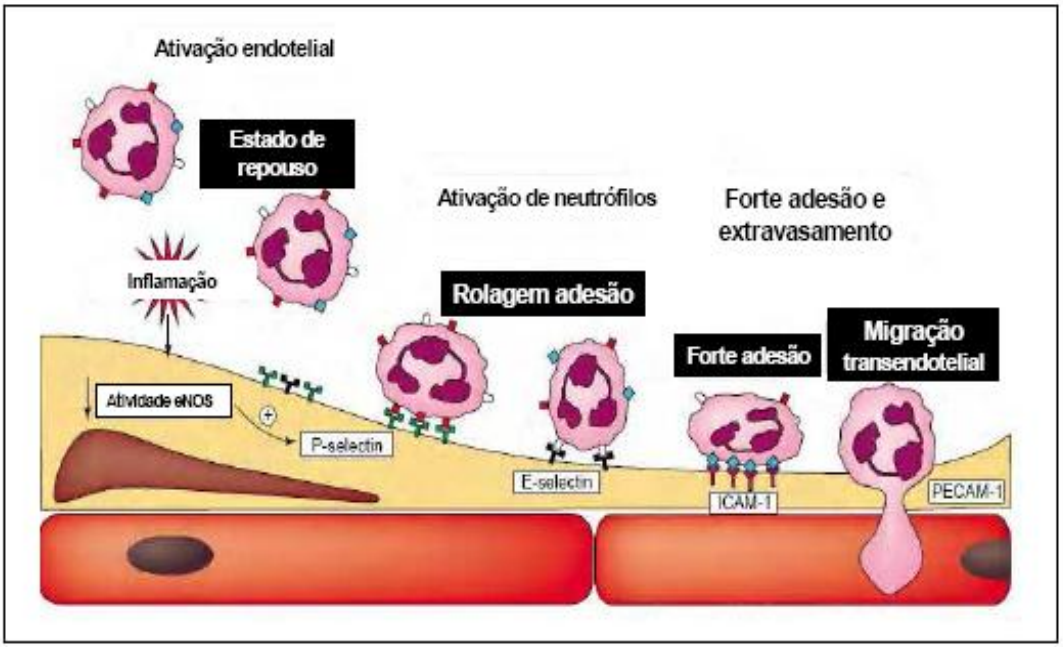

Figura 2: Interações entre os leucócitos e a expressão das moléculas de adesão no vaso. Rolamento, adesão, migração transendotelial, selectinas, integrinas e ligantes das moléculas de adesão (Adaptado de Kakkar et al., 2004).

1.7. Neuroinflamação

A inflamação associada ao SNC, denominada de neuroinflamação, difere da encontrada em outros tecidos e órgãos periféricos. É importante salientar que a resposta imunológica dentro do encéfalo é limitada e peculiar, condição essa que o levou a ser descrito como ‘’órgão imunologicamente privilegiado”,

um privilégio garantido pela permeabilidade seletiva da barreira hematoencefálica que separa o cérebro da periferia, prevenindo não somente agentes nocivos como também leucócitos circulantes e anticorpos de entrarem no cérebro. Sabe-se que essa é uma verdade parcial, uma vez que em determinadas situações pode-se observar a infiltração de outras células (Engelhardt e Ransohoff, 2005). Na imensa maioria dos casos, porém, apenas a microglia e os astrócitos são ativados, sendo esses dois tipos celulares, em especial o primeiro, os responsáveis pela resposta imunológica no SNC (Liberto et al., 2004; Rock et al., 2004). As células microgliais são de origem mesodérmica e são consideradas os macrófagos que residem no SNC. Estão localizadas no parênquima tecidual, próximas aos vasos, em todas as regiões do SNC, correspondendo a 10-20% das células gliais. Devido a sua plasticidade e reatividade a um amplo espectro de estímulos, parecem possuir um papel importante na defesa do SNC, na neuroproteção e no reparo do tecido pós-lesões, uma vez que podem migrar para os locais de dano tecidual (Minghetti e Levi, 1998; Rock et al., 2004). Essas células podem ser facilmente reconhecidas no tecido pela marcação com anticorpos específicos como o anti-CD11b (Strassburger et al., 2008) e anti-lipocortina (LC1) (Skibo et al., 2000). Dentre as substâncias secretadas pela microglia ativada podemos citar o glutamato, importante neurotransmissor excitatório que quando em elevadas concentrações pode levar à excitotoxicidade (Takeuchi et al., 2006), fatores de crescimento como o fator de crescimento tumoral beta (TGF-β), citocinas como a interleucina-1β (IL-1β), interleucina 6 (IL-6) e o fator de necrose tumoral alfa (TNF-α), EROS, NO, além de quimiocinas, mediadores lipídicos, fatores de coagulação e componentes da matrix extracelular. Além disso, na forma

ativada, a microglia aumenta a produção de ciclooxigenase-2 (COX-2), participando ativamente na síntese de prostanóides como prostaglandinas, leucotrienos e tromboxanos, outros importantes mediadores da resposta inflamatória (Minghetti e Levi, 1998; Rock et al., 2004). A participação da microglia na morte neuronal pode ser demonstrada na Figura 3.

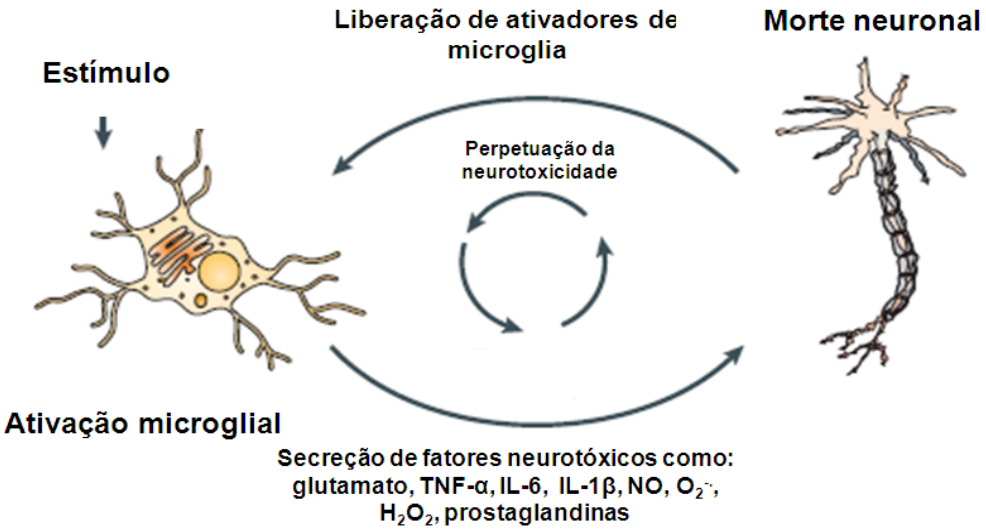

Figura 3: Participação da ativação da microglia na morte neuronal (Adaptado de Block et al., 2007).

1.8. Mediadores envolvidos na inflamação

A resposta de fase aguda é uma reação inflamatória inespecífica que ocorre pouco depois de qualquer dano tecidual. Esta resposta inclui mudanças na concentração de proteínas plasmáticas, chamadas de proteínas de fase aguda. Algumas delas diminuem sua concentração (proteínas de fase aguda negativas), tais como a albumina e a transferrina, e outras aumentam (proteínas de fase aguda positivas) como, por exemplo, proteína C reativa

(PCR), α_1- glicoproteína ácida, haptoglobina e ceruloplasmina. A maioria das proteínas de fase aguda positivas são glicoproteínas sintetizadas principalmente pelos hepatócitos sob estimulação de citocinas pró-inflamatórias como o TNF-α, IL-1β e IL-6, sendo posteriormente lançadas na corrente circulatória (Ceron et al., 2005).

Outro mediador extremamente importante na resposta inflamatória são as citocinas. As citocinas são pequenas proteínas, com peso molecular de 8 a 40 kDa, que podem ser produzidas em todos os tecidos e pela maioria das células dos sistemas imune inato e adaptativo (Chiche et al., 2001). Estas são sintetizadas em resposta a estímulos inflamatórios ou antigênicos. Em geral atuam localmente, de modo parácrino ou autócrino, pela ligação a receptores de alta afinidade nas células-alvo. Algumas citocinas também podem ser produzidas em quantidades suficientes para circular e exercer funções endócrinas (Abbas et al., 2003).

Dentre as diversas citocinas pró-inflamatórias, o TNF-α possui um importante papel inicial na resposta inflamatória. O TNF-α pode interagir com células endoteliais e induzi-las à expressão de moléculas de adesão como a molécula de adesão intercelular (ICAM-1), molécula de adesão vascular (VCAM-1) e selectina E, permitindo o acesso de granulócitos ao foco da inflamação. Essa citocina é um potente ativador de neutrófilos mediando a aderência, a quimiotaxia, a desgranulação e a explosão oxidativa ("*oxidative burst*"), além de também ser capaz de causar alterações de permeabilidade vascular (Hehlgans e Pfeffer, 2005). Os aspectos moleculares das interações entre o TNF-α e seus receptores são bastante complexos, ocorrendo ativação de diversos mecanismos intracelulares como a ativação de caspases (Kischkel

et al., 2000), fator de transcrição nuclear-kapaB (NF-kB), proteína cinase de C-Jun n-terminal (JNK) (Stanger et al., 1995; Hsu et al., 1996), cinase regulada por sinais extracelulares (ERK) e fosfatidilinositol-3-cinase (PI3K) (Dempsey et al., 2003).

A IL-1β é uma citocina presente na resposta imune inata. É produzida por macrófagos, neutrófilos, células epiteliais e células endoteliais frente a produtos bacterianos, como o lipopolissacarídeo (LPS) e outras citocinas como o TNF-α (Kondera-Anasz et al., 2005). Em baixas concentrações, a IL-1β participa da inflamação local pela ação sobre células endoteliais, aumentando o número de moléculas de superfície para a adesão leucocitária. Em grandes concentrações passa a ter um efeito sistêmico, causando febre e produção de proteínas de fase aguda pelo fígado (Cao et al., 2005). Outra citocina com papel central na resposta inflamatória é a IL-6. A IL-6 faz parte dos processos da imunidade inata e da adquirida, produzida em resposta ao TNF-α e a IL-1β e a algumas células T ativadas. Na resposta inata, a IL-6 estimula a síntese de proteínas de fase aguda pelo fígado; já na imunidade adquirida, a IL-6 estimula o desenvolvimento dos linfócitos B produtores de anticorpos (Hirota et al., 2005).

As quimiocinas pertencem a um restrito grupo de citocinas de 8 a 12 kDa, que foram inicialmente identificadas devido à sua habilidade em atrair leucócitos (Wallace et al., 2004). Elas são produzidas por uma variedade de células incluindo: neutrófilos, monócitos, linfócitos, eosinófilos, fibroblastos e queratinócitos (Borish e Steinke 2003), células endoteliais (Briones et al., 2001), células epiteliais (Sexton e Walsh, 2002), células dendríticas (Vulcano et al., 2001) e células T (Matsumoto et al., 1998). As quimiocinas contribuem para

a resposta inflamatória dada sua capacidade de induzir recrutamento e ativar populações de leucócitos, induzir degranulação e levar à liberação de mediadores inflamatórios de células efetoras, tais como basófilos, mastócitos, neutrófilos e eosinófilos. A família de quimiocinas CC é a mais diversa e numerosa; dentre elas, podemos salientar a proteína quimiotática de monócito do tipo 1 (MCP1) (Locati et al., 2002).

Além das proteínas de fase aguda, citocinas e quimiocinas, outro mediador bastante importante durante o processo inflamatório é o NO. A molécula de NO é um radical livre (apresenta um elétron desemparelhado na órbita externa), em estado gasoso que difunde livremente e permeia membranas rapidamente. Sua meia vida é de aproximadamente 3 a 5 segundos, tão curta que se torna muito difícil sua mensuração *in vivo*. Produtos de sua oxidação, nitritos e nitratos, são os principais metabólitos que podem ser usados como indicadores da produção de NO (Orida e Lai, 2000).

O NO é produzido através da conversão do aminoácido, L-arginina à L-citrulina pela ação da enzima óxido nítrico sintase (NOS). A isoforma da NOS de expressão constitutiva (cNOS) é, basicamente, encontrada nas células endoteliais (denominada eNOS, ou tipo I), nos neurônios (nNOS, ou tipo III) e, também, nas células epiteliais, neutrófilos e plaquetas, produzindo NO em quantidades fisiológicas. O terceiro tipo de isômero, denominado de induzível (iNOS) ou NOS tipo II, expressa-se sob a ação de citocinas, endotoxinas, e outros mediadores inflamatórios, e se expressa, principalmente, em neutrófilos, macrófagos, fibroblastos, células endoteliais e musculatura lisa dos vasos, mediando a produção de grandes quantidades de NO (Moncada e Higgs, 1993; Moncada et al., 2002). Agindo autócrina ou paracrinamente, o NO está

envolvido no relaxamento do músculo liso, diminuição da agregação plaquetária, sinalização celular, aprendizado, além de respostas imunológicas citotóxicas (Palmer e Higgs, 2002).

O NF-kB é um regulador importante na expressão de genes responsáveis pela síntese de citocinas pró-inflamatórias, quimocinas, moléculas de adesão, além de mediadores envolvidos na inflamação (Tak e Firestein, 2001). Inicialmente, a função fisiológica do NF-kB foi descrita primeiramente como sendo um regulador da expressão da cadeia leve das imunoglobulinas (cadeia kappa) em células B. Hoje em dia, sabe-se que o NF-kB pré-existe no citoplasma na maioria das células de origem mesenquimal na sua forma inativada, ligada a proteínas inibitórias específicas denominadas de IkB (Ghosh et al., 1998; Karin e Bem-Neriah, 2000). A partir de um estímulo pró-inflamatório, o NF-kB libera-se das proteínas inibitórias (IkB), desloca-se para o núcleo onde se liga a sequências promotoras de genes, produzindo a síntese de mediadores pró-inflamatórios (Handel et al., 2000), incluindo moléculas de adesão, enzimas, citocinas, entre outros (Christman et al., 2000).

Há também a participação de mediadores lipídicos na resposta inflamatória. O ácido araquidônico (AA) é liberado da membrana lipídica após vários estímulos, tais como: físicos, químicos, citocinas, dentre outros. A seguir, o AA é mobilizado da membrana fosfolipídica pela ação de fosfolipases, liberando uma variedade de metabólitos biologicamente ativos (Rocca e Fitzgerald, 2002). Como derivam de um ácido graxo eicosanóico, esses compostos: tromboxano A_2, prostaglandinas, epoxigenases, leucotrienos e lipoxinas são coletivamente denominados de eicosanóides. Os eicosanóides medeiam uma enorme variedade de interações em processos fisiológicos e

patológicos envolvidos na homeostasia, na trombose, na filtração glomerular e no balanço hidrostático, na ovulação, na implantação embrionária e desenvolvimento, na iniciação do trabalho de parto/aborto, na inflamação e modulação de respostas imunológicas, entre outros (Rocca e Fitzgerald, 2002). As prostaglandinas são ainda produzidas no SNC, em vasos e neurônios, participando dos mecanismos de sono e despertar; e mais recentemente, têm-se estudado sua participação no processo de doenças como na Esclerose Múltipla e na Doença de Alzheimer (Rocca e Fitzgerald, 2002).

A acetilcolina (ACh) é um neurotransmissor clássico e recentemente tem sido associado à inflamação (Zimmerman e Soreq, 2006; Tracey, 2007). É sintetizado pela enzima colina acetiltransferase a partir de acetato e colina, e armazenado em vesículas no neurônio pré-sináptico. A atividade colinérgica é controlada principalmente pela enzima acetilcolinesterase (AChE) que hidrolisa rapidamente a ACh nas sinapses colinérgicas e junção neuromuscular finalizando a transmissão colinérgica (Zimmerman e Soreq, 2006).

Diversos estudos demonstraram a expressão de ACh também em células não neuronais, desenvolvendo-se assim o conceito de sistema colinérgico não neuronal. Vários dos componentes colinérgicos têm sido identificados em vários tipos celulares como em células epiteliais, endoteliais e mesoteliais e vários tipos de leucócitos (linfócitos, macrófagos, eosinófilos e neutrófilos) (Kirkpatrick et al., 2003). A via colinérgica anti-inflamatória representa um mecanismo de resposta do SNC à presença de estímulos inflamatórios na circulação sendo mediada pela ação do nervo vago (Tracey, 2007). Citocinas pró-inflamatórias como a IL-1β ativam as fibras aferentes do nervo vago, as quais servem de sensor para a inflamação (Pavlov e Tracey,

2005; Gwilt et al., 2007). Esta informação é transmitida ao SNC, o qual estimula o nervo vago eferente para a produção de ACh, que induz então a inibição da síntese e liberação de citocinas pró-inflamatórias por macrófagos e outras células produtoras de citocinas (Gwilt et al., 2007; Parrish et al., 2008). Assim, a via colinérgica anti-inflamatória representa um mecanismo fisiológico pelo qual o sistema nervoso inter-atua com o sistema imune inato para controlar a resposta inflamatória (Gallowitsch-Puerta e Pavlov, 2007). Os diferentes mediadores secretados durante a resposta inflamatória podem ser visualizados na Figura 4.

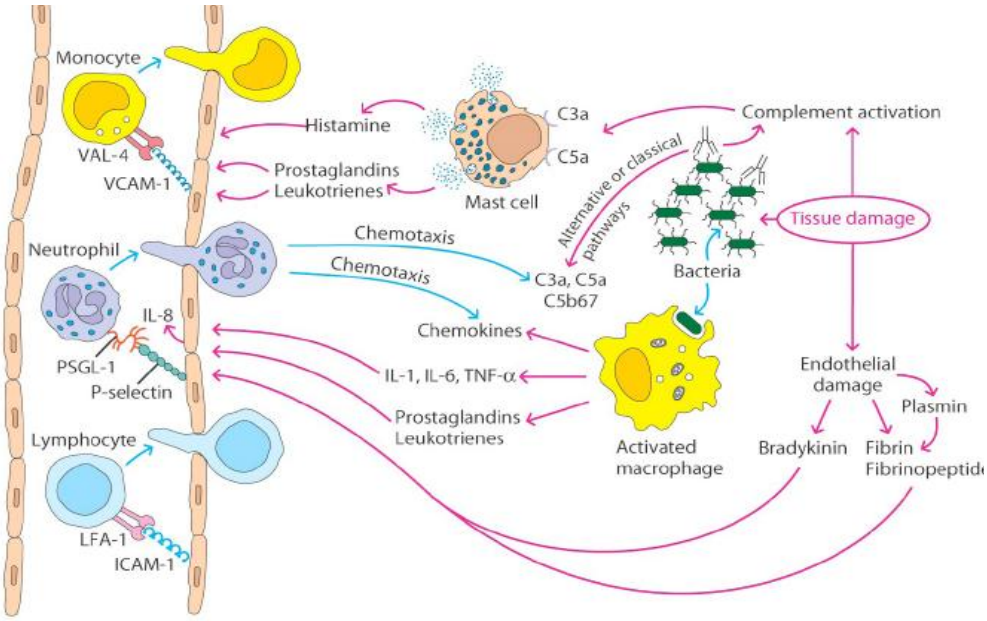

Figura 4: Diferentes mediadores secretados durante a resposta inflamatória (Adaptado de Delves e Roitt, 2000).

1.10. Coagulação sanguínea

Junto à ativação do sistema inflamatório, o sistema de coagulação também é recrutado (Aird, 2001). A formação do coágulo de fibrina no sítio da

lesão endotelial constitui um processo crucial para a manutenção da integridade vascular. Os mecanismos operantes nesse processo são dependentes da integridade anatômica e funcional do sistema hemostático, e devem ser finalmente regulados de modo a evitar a perda excessiva de sangue e a formação de trombos intravasculares decorrentes da formação excessiva de fibrina. Os componentes do sistema hemostático incluem as plaquetas, os vasos, as proteínas da coagulação do sangue, os anticoagulantes naturais e o sistema de fibrinólise. O equilíbrio funcional desses fatores é garantido por uma variedade de mecanismos envolvendo interações entre proteínas, respostas celulares complexas, e regulação de fluxo sanguíneo (Stassen et al., 2004; Moran e Viele, 2005).

A formação do coágulo de fibrina envolve complexas interações entre proteases plasmáticas e seus cofatores, que culminam na gênese da trombina, que por proteólise converte o fibrogênio solúvel em fibrina insolúvel (Seré e Hackeng, 2003; Stassen et al., 2004). Em 1964, Macfarlane propôs a hipótese da ‘’cascata’’ para explicar a fisiologia da coagulação no sangue. Nesse modelo, a coagulação ocorre por meio da ativação proteolítica sequencial de zimógenos por proteases plasmáticas, resultando na formação de trombina, que então converge o fibrinogênio em fibrina. A coagulação se dividiria em uma via extrínseca e uma via intrínseca, que convergem no ponto de ativação do fator X (‘’via final comum’’). Na via extrínseca, o fator VII plasmático ativa diretamente o fator X. Na via intrínseca, ativação do fator XII ocorre por contato e requer ainda a presença de pré-calicreína e cininogênio de alto peso molecular. O fator XIIa ativa o fator XI, que por sua vez ativa o fator IX. O fator IXa, na presença do fator VIII, ativa o fator X da coagulação, desencadeando a

geração de trombina e subsequente formação de fibrina (Macfarlane, 1964; Yarovaya et al., 2002; Moran e Viele, 2005), conforme demostrado na Figura 5.

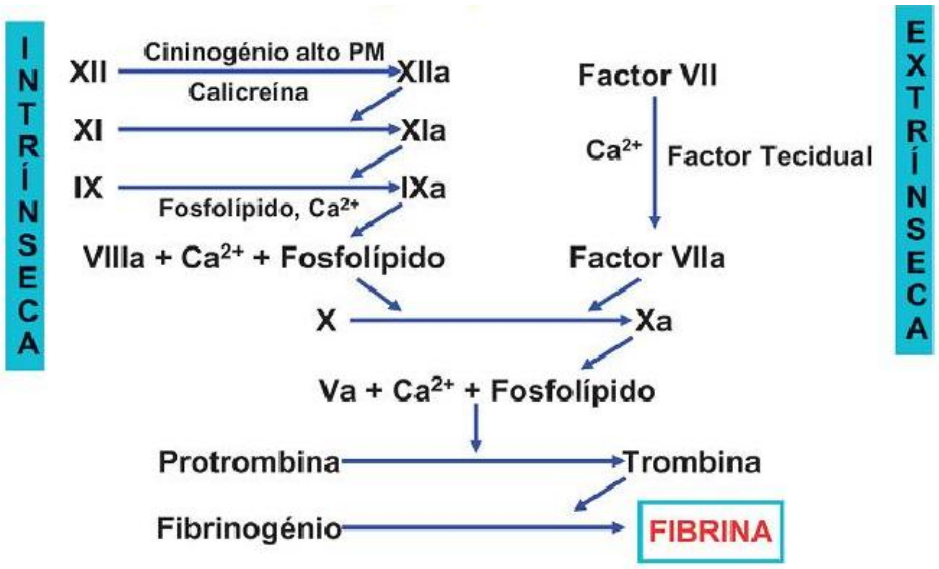

Figura 5: Representação da cascata da coagulação (Adaptado de Kurata e Horii, 2004).

Embora tradicionalmente ainda se observe a divisão do sistema de coagulação do sangue em intrínseco e extrínseco, essa divisão, atualmente, parece ser inadequada para o entendimento da fisiologia da coagulação. A análise do conjunto de reações envolvidas na coagulação do sangue mostra que não há distinção clara entre os sistemas intrínseco e extrínseco, que atuam de modo altamente interativo *in vivo*. No entanto, a utilização dos termos "intrínseco" e "extrínseco'' é útil para fins didáticos e na interpretação de exames laboratoriais utilizados na rotina da avaliação da hemostasia: o tempo de protrombina (TP que avalia a via extrínseca) e o KTTP (tempo de tromboplastina parcial ativada que avalia a via intrínseca), que são de particular

importância na monitorização da terapêutica anticoagulante (Kurata e Horii, 2004).

1.11. Vias de transdução de sinal

De uma forma simplificada, o mecanismo de transdução de sinal utilizado por uma célula é uma interação entre proteínas específicas em uma ordem determinada. Sabemos que os princípios moleculares nos quais a transdução de sinal se baseia são representados por associação específica de proteínas e sua fosforilação (ou desfosforilação). Assim, o balanço entre fosforilação e desfosforilação é determinante para a transdução de sinal intracelular (Cooper et al., 2001; López-Neblina e Toledo-Pereyra, 2006).

A produção de citocinas desempenha um importante papel na resposta imune e estudos relacionam que a liberação de diferentes citocinas está intimamente associada com a via de sinalização da fosfatidilinositol-3-cinase (PI3K) (Goyal et al., 2002). A PI3K é uma enzima amplamente expressa que regula vários processos celulares, como proliferação, crescimento e apoptose e diversos receptores de superfície, especialmente os ligados à tirosina cinase, podem ativar a PI3K (Datta et al., 1999). Um dos alvos do segundo mensageiro gerado pela ativação da PI3K é a proteína Akt (Datta et al., 1999).

A Akt, também conhecida como Proteína cinase B (PKB) é uma serina/treonina quinase de 60 kDa (Datta et al., 1999) envolvida com a regulação da proliferação e sobrevivência celular (Song et al., 2005). A ativação da Akt é um processo que envolve várias etapas e proteínas adicionais. A ativação da PI3K por fatores de crescimento resulta em um

aumento do PIP3, que leva a translocação da Akt para a membrana e uma modificação conformacional que permite que a cinase dependente de fosfoinositol-1 (PDK-1) e a cinase dependente de fosfoinositol-2 (PDK-2) fosforilem os resíduos Ser473 e Thr308, ativando totalmente a Akt (Datta et al., 1997; Coffer et al., 1998).

A Akt é responsável pela fosforilação de vários substratos citosólicos e nucleares que regulam o metabolismo e o crescimento celular. A Akt é capaz de regular proteínas envolvidas no processo de apoptose (Datta et al., 1997; Coffer et al., 1998; Yuan e Yankner, 2000), por interagir com diferentes proteínas, tais como BAD (mantendo-a fosforilada e impedindo com que essa atue sobre a mitocôndria provocando a liberação do citocromo c e consequente disparo da apoptose (Datta et al., 1997); caspase 9 (fosforilando-a e impedindo que ela seja clivada, impedindo a clivagem e ativação da caspase 3 (Cardone et al., 1998); glicogênio sintase cinase-3 beta (GSK-3β) (fosforilando-a e inativando-a para também por essa via bloquear a morte celular) (Pap e Cooper, 1998). Além disso, a Akt é capaz de interagir com a proteína inibitória IkB do NF-κB fosforilando-a e liberando o fator de transcrição NF-κB, estimulando a secreção de citocinas pró-inflamatórias (Ozes et al., 1999). Também é capaz de fosforilar a eNOS, ativando-a e levando à produção de NO, um importante sinalizador celular (Fulton et al., 1999). As interações da via da Akt podem ser melhor visualizadas na figura 6.

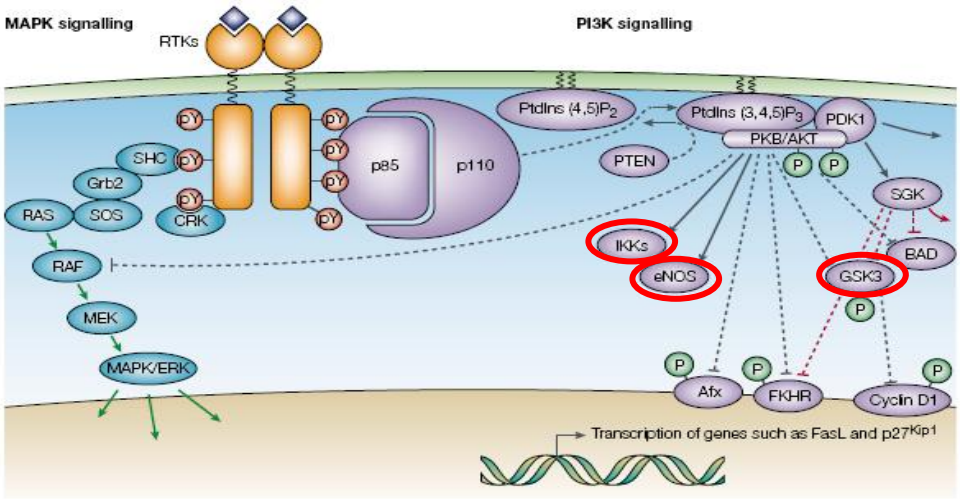

Figura 6: Vias de sinalização da Akt. As setas inteiras indicam que as proteínas alvo estão sendo ativadas, enquanto que as setas tracejadas indicam que as proteínas alvo estão sendo inibidas (Adaptado de Scheid e Woodget, 2010).

A GSK-3β, encontrada abundantemente no SNC, particularmente em neurônios é uma serina/treonina cinase inicialmente descrita como uma peça chave no metabolismo do glicogênio pela sua habilidade de fosforilar e inativar a glicogênio sintase; atualmente, sabe-se que ela regula diversas funções celulares. A GSK-3β encontra-se ativa quando desfosforilada, mas pode ser inibida através do aumento da fosforilação no sítio específico da Ser9 pela Akt, pela proteína cinase C (PKC), pela proteína cinase A (PKA), proteína quinase ativada por mitógenos (MAPK), entre outras. Várias são as funções biológicas mediadas pela GSK-3β, como por exemplo, a regulação de proteínas do metabolismo e sinalização celular (incluindo ciclina D1 e *nerve growth factor-NGF*), proteínas estruturais (incluindo neurofilamentos e *Tau*) e fatores de transcrição (Cohen e Frame, 2001; Grimes e Jope, 2001). A GSK-3β é também considerada uma enzima pró-apoptótica por inibir uma variedade de fatores de transcrição importantes para a sobrevivência celular (Crespo-Biel et al., 2007).

Dentre as diversas funções reguladas pela GSK-3β, recentemente foi identificado um papel da GSK-3β na modulação da inflamação por promover a secreção de diferentes citocinas (TNF-α, IL-1β e IL-6) e estimular a migração celular. O uso de inibidores da GSK-3β em diferentes modelos animais de doenças inflamatórias parece apresentar um efeito anti-inflamatório, sendo considerado uma promissora estratégia terapêutica no controle da inflamação. Entretanto, os mecanismos desses efeitos ainda não estão completamente compreendidos (Martin et al., 2005).

A GSK-3β é considerada a principal cinase na regulação da fosforilação da proteína *Tau* no cérebro. Os emaranhados neurofibrilares são constituídos principalmente pela deposição de filamentos helicoidais pareados da proteína *Tau* hiperfosforilada. A proteína *Tau* é responsável por estabilizar os microtúbulos do citoesqueleto neuronal, sendo essa função regulada por um processo de fosforilação e desfosforilação. Além disso, sabemos que o citoesqueleto possui a função chave de manter a forma e a polaridade estrutural dos neurônios, essenciais para a fisiologia neuronal (Ferrari et al., 2003; Golde, 2007; Hooper et al., 2008).

1.12. Estresse oxidativo

Radical livre é definido como uma espécie química que contém um ou mais elétrons desemparelhados, o que os torna altamente reativos (Halliwell e Gutteridge, 2007). São exemplos de EROS e de espécies reativas de nitrogênio (ERNS) os radicais $O_2^{\bullet -}$, $OH^{\bullet}$ e NO; e os não radicais H_2O_2, oxigênio singlet ($^1O_2$) e peroxinitrito ($ONOO^-$) (Halliwell e Whiteman, 2004).

As espécies reativas são constantemente produzidas no organismo em níveis basais, principalmente durante o processo de respiração celular, através da redução incompleta do oxigênio molecular (que ocorre de 2 a 5%). Essas espécies reativas desempenham funções fisiológicas importantes como fagocitose, sinalização celular, regulação de proteínas e plasticidade sináptica (Halliwell e Gutteridge, 2007). Porém, quando em excesso, as espécies reativas induzem o estresse oxidativo, o qual é definido como um desequilíbrio entre os níveis de espécies reativas e das defesas antioxidantes, que pode ser resultado tanto do aumento da produção dessas espécies, como da diminuição dos níveis de antioxidantes ou da combinação de ambos (Halliwell e Gutteridge, 2007). O estresse oxidativo pode causar dano em diferentes tipos de biomoléculas, incluindo o DNA, proteínas e lipídios (Halliwell e Whiteman, 2004; Halliwell e Gutteridge, 2007), conforme demonstrado na Figura 7.

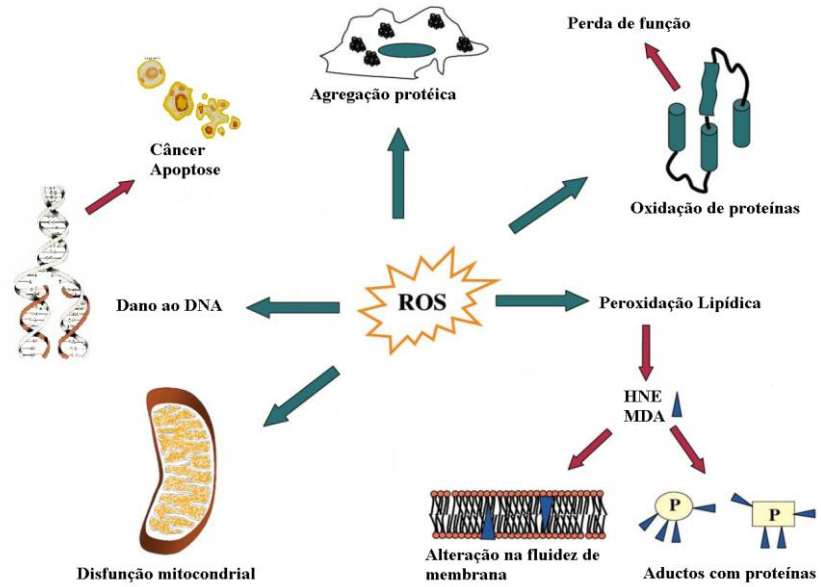

Figura 7: Dano oxidativo às biomoléculas (Adaptado de Halliwell e Gutteridge, 2007).

O organismo possui mecanismos de defesa contra a ação tóxica dos radicais livres e das espécies reativas, diminuindo ou eliminando as consequências negativas de seus efeitos no organismo. Esses mecanismos compreendem as defesas antioxidantes que são classificadas em enzimáticas e não-enzimáticas (Halliwell e Gutteridge, 2007). As defesas antioxidantes enzimáticas incluem, principalmente, as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) (Halliwell e Gutteridge, 2007) e as defesas antioxidantes não-enzimáticas incluem substâncias como a glutationa reduzida (GSH), ácido ascórbico (vitamina C), α-tocoferol (vitamina E), melatonina, urato entre outras (Halliwell e Gutteridge, 2007). A SOD é uma metaloenzima que catalisa a dismutação do $O_2^{\bullet-}$, formando H_2O_2 e O_2 **($O_2^{\bullet-}$ + $O_2^{\bullet-}$ + $2H^+$ → H_2O_2 + O_2)** (Halliwell e Gutteridge, 2007). A dismutação do $O_2^{\bullet-}$ catalisada pela SOD gera H_2O_2, que será decomposto diretamente a O_2 por ação subsequente da enzima antioxidante CAT. O mecanismo de reação da CAT é, assim como a SOD, essencialmente uma dismutação; uma molécula de H_2O_2 é reduzida a H_2O e a outra é oxidada a O_2 **($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$)**. A GPx também atua decompondo o H_2O_2, através do acoplamento de sua redução a H_2O com a concomitante oxidação da GSH ao dissulfeto de glutationa (GSSG) **(H_2O_2 + 2GSH → GSSG + $2\ H_2O$)** (Chance et al., 1979; Wendel, 1981; Halliwell e Gutteridge, 2007).

O estresse oxidativo, assim como a inflamação, pode possuir um papel benéfico ou deletério, dependendo da sua intensidade (Valko et al., 2007), e parece estar envolvido na patogênese do dano neurológico de várias doenças que afetam o SNC (Halliwell e Gutteridge, 2007). Além disso, dados na literatura demonstram o envolvimento da geração de espécies reativas em

várias doenças pulmonares, como na doença pulmonar obstrutiva crônica, na lesão pulmonar aguda e na asma (Al-Mehdi et al., 1997; da Cunha et al., 2011; Kirkham et al., 2011).

2. OBJETIVOS

2.1. Objetivo geral

A presente tese de doutorado tem como objetivo geral avaliar o efeito da hiper-homocisteinemia severa sobre mediadores inflamatórios e sobre o estresse oxidativo em cérebro, sangue e pulmão de ratos.

2.2. Objetivos específicos:

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

- **Capítulo I**

- Investigar os níveis das citocinas (TNF-α, IL-1β e IL-6) e da quimiocina CCL_2 (MCP-1) no hipocampo e no córtex cerebral de ratos submetidos à hiper-homocisteinemia aguda severa;

- Investigar os níveis das citocinas (TNF-α e IL-6) no soro de ratos submetidos à hiper-homocisteinemia aguda severa;

- Analisar os níveis de nitritos no hipocampo e no córtex cerebral de ratos submetidos à hiper-homocisteinemia aguda severa;

- Avaliar os níveis das proteínas de fase aguda (PCR e α_1-glicoproteína ácida) no soro de ratos submetidos à hiper-homocisteinemia aguda severa;

- Determinar a contagem diferencial de leucócitos no sangue de ratos submetidos à hiper-homocisteinemia aguda severa.

➢ **Capítulo II**

- Investigar os níveis das citocinas (TNF-α, IL-1β e IL-6) e da quimiocina CCL_2 (MCP-1) no hipocampo e no soro de ratos submetidos à hiper-homocisteinemia crônica severa;

- Avaliar os níveis de nitritos no hipocampo e no soro de ratos submetidos à hiper-homocisteinemia crônica severa;

- Determinar os níveis de prostaglandina E_2 no hipocampo e no soro de ratos submetidos à hiper-homocisteinemia crônica severa;

- Analisar o imunoconteúdo das frações citoplasmática e nuclear da subunidade p65 do NF-κB no hipocampo de ratos submetidos à hiper-homocisteinemia crônica severa;

- Investigar a atividade da acetilcolinesterase no hipocampo de ratos submetidos à hiper-homocisteinemia crônica severa.

- **Capítulo III**

- Investigar o efeito da administração aguda de Hcy sobre parâmetros da coagulação sanguinea (contagem de plaquetas, TP, KTTP e fibrinogênio) no sangue e/ou plasma de ratos submetidos à hiper-homocisteinemia aguda severa;

- Analisar os níveis de nitritos no plasma de ratos submetidos à hiper-homocisteinemia aguda severa;

- Determinar alguns parâmetros de estresse oxidativo, tais como os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), atividade das enzimas antioxidantes (SOD, CAT e GPx) no plasma e/ou eritrócitos de ratos submetidos à hiper-homocisteinemia aguda severa.

- **Capítulo IV**

- Investigar as vias de sinalização inflamatórias da Akt e GSK-3β no hipocampo de ratos submetidos à hiper-homocisteinemia aguda e crônica severa;

- Analisar o imunoconteúdo das frações citoplasmática e nuclear da subunidade p65 do NF-κB no hipocampo de ratos submetidos à hiper-homocisteinemia aguda severa;

- Investigar o efeito da fosforilação da proteína *Tau* no hipocampo de ratos submetidos à hiper-homocisteinemia aguda severa.

- **Capítulo V**

- Investigar alguns parâmetros de estresse oxidativo, tais como TBARS (um índice de lipoperoxidação) e carbonilas (uma medida de dano a proteínas) no pulmão de ratos submetidos à hiper-homocisteinemia crônica severa;

- Verificar os níveis de espécies reativas (oxidação do DCF) e o potencial antioxidante total não-enzimático (TRAP) no pulmão de ratos submetidos à hiper-homocisteinemia crônica severa;

- Determinar a atividade das enzimas antioxidantes (SOD, CAT e GPx) no pulmão de ratos submetidos à hiper-homocisteinemia crônica severa;

- Determinar o nível da glutationa reduzida (GSH) e a atividade da enzima glicose-6-fosfato desidrogenase (G6PD) no pulmão de ratos submetidos à hiper-homocisteinemia crônica severa;

- Analisar os níveis de nitritos no pulmão de ratos submetidos à hiper-homocisteinemia crônica severa.

PARTE II

1. METODOLOGIA E RESULTADOS

1.1. Modelos experimentais

Os capítulos I, II, III, IV e V serão apresentados na forma de artigos científicos, os quais apresentam desenhos experimentais semelhantes entre si no que se refere aos modelos de hiper-homocisteinemia aguda ou crônica severa.

1.1.2. *Modelo experimental de hiper-homocisteinemia aguda severa:* Ratos Wistar machos de 29 dias foram submetidos a uma única administração subcutânea de Hcy (0,6 µmol/g peso corporal) e foram eutanaziados 15 minutos, 1, 6 ou 12 horas após a administração de Hcy. Os animais do grupo controle receberam o mesmo volume de solução salina (Streck et al., 2002).

1.1.3. Modelo experimental de *hiper-homocisteinemia* crônica severa: Ratos Wistar foram submetidos a duas injeções subcutâneas diárias de Hcy do 6º ao 28º dia de vida (0,3-0,6 µmol/g peso corporal) e foram eutanaziados 1 ou 12 horas após a última injeção de Hcy. Os animais do grupo controle receberam o mesmo volume de solução salina (Streck et al., 2002).

Capítulo I

Metab Brain Dis
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ORIGINAL PAPER

Increased inflammatory markers in brain and blood of rats subjected to acute homocysteine administration

Aline A. da Cunha · Andréa G. K. Ferreira · Angela T. S. Wyse

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Abstract Hyperhomocysteinemia plays an etiologic role in the pathogenesis of disorders, including homocystinuria and neurodegenerative and cardiovascular diseases. In the present study, we studied the effect of acute administration of homocysteine, similar to that found in homocystinuria, on parameters of inflammation such as cytokines (TNF-α, IL-1β and IL-6), chemokine CCL2 (MCP-1), nitrite and acute phase-proteins (C-reactive protein and α_1-Acid glycoprotein) levels in brain and blood of rats. In addition, a differential count of blood leukocytes was performed. Wistar rats, aged 29 days, received a single subcutaneous injection of saline (control) or homocysteine (0.6 μmol/g body weight). Fifteen minutes, 1 h, 6 h or 12 h after the injection, the rats were sacrificed and serum, hippocampus and cerebral cortex were used. Results showed that homocysteine significantly increased proinflammatory cytokines (TNF-α, IL-1β and IL-6) and chemokine CCL2 (MCP-1) in serum, hippocampus and cerebral cortex. Nitrite levels also increased in hippocampus and cerebral cortex at 15 min, 1 h and 6 h, but not 12 h after homocysteine administration. Acute phase-protein levels were not altered by homocysteine. The percentage of neutrophils and monocytes significantly increased in blood at 15 min and 1 h, but not at 6 h and 12 h after acute hyperhomocysteinemia, when compared to the control group. Our results showed that acute administration of homocysteine increased inflammatory parameters, suggesting that inflammation might be associated, at least in part, with the neuronal and cardiovascular dysfunctions observed in homocystinuric patients.

Keywords Homocysteine · Cytokines · Chemokine · Acute-phase proteins and nitrite

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Introduction

Elevated homocysteine (Hcy) levels can be found in several disorders, such as homocystinuria, and neurodegenerative and neuroinflammatory diseases (Mudd et al. 2001; Tyagi et al. 2009). Homocystinuria is an inborn error of metabolism caused by severe deficiency of cystathionine β-synthase (CBS, EC 4.2.1.22) activity. Affected patients present alterations in various organs and systems, especially the central nervous and the vascular systems and present manifestations that include mental retardation, seizures and atherosclerosis (Mudd et al. 2001), whose underlying mechanisms are still obscure.

Inflammation often elicits a generalized sequence of events, known as the acute phase response (Suffredini et al. 1999), which is mediated by the generation of early response cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-alpha (TNF-α), acute-phase proteins, expression of cell-surface adhesion molecules, as well as chemotactic molecules production (Keane and Strieter 2000). Although several cell types of the central nervous system (CNS) are able to secrete cytokines including microglia, astrocytes and neurons, there is evidence that peripherally-derived cells can contribute to brain inflammation and injury (Ghirnikar et al. 1998; Giulian et al. 1989). It has been shown that cytokines cross the blood-brain barrier (BBB), probably either by active transport or through leaky regions of endothelia, when BBB is

compromised by a pathological condition. Thus, the CNS can be affected not only by inflammatory mediators produced in the brain, but also by the actions of mediators from the periphery (Pollmacher et al. 2002).

It has been shown that exposure of cultured endothelial cells (ECs) to Hcy leads to endothelial activation, resulting in the increased expression of chemokines (Poddar et al. 2001) and adhesion molecules (Wang et al. 2002). On the other hand, Upchurch et al. (1997) suggest that endothelial injury, caused by Hcy, may be due to oxidative stress, nitric oxide (NO) and disturbances in the anti-thrombotic activities of the endothelium. In this context, we have previously reported that an acute experimental model of hyperhomocysteinemia in rats, similar to that found in homocystinuria, induces oxidative stress, reducing antioxidant defenses and increasing lipid peroxidation (Wyse et al. 2002; Matté et al. 2004, 2009).

In order to verify whether high Hcy levels could alter inflammatory markers, in the present study we evaluated the effect of acute Hcy administration on inflammatory parameters such as cytokines (TNF-α, IL-1β and IL-6), chemokine CCL2 (MCP-1), nitrite and acute-phase proteins [(C-reative protein (CRP) and α_1-Acid glycoprotein)] levels in hippocampus, cerebral cortex and serum. Differential leukocytes counts in the blood of rats were also performed.

Materials and methods

Animals and reagents

Sixty-five Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant room 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. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Acute homocysteine treatment

Wistar rats, aged 29 days, received a single subcutaneous injection of saline solution (control) or Hcy (0.6 μmol/g body weight). *D*,*L*-Hcy was dissolved in 0.9% NaCl solution (saline) and buffered to pH7.4. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those found in homocystinuric patients (Streck et al. 2002; Matté et al. 2009; Mudd et al. 2001). Rats were sacrificed by decapitation without anesthesia 15 min, 1 h, 6 h or 12 h after the injection; serum was separated and brain was quickly removed and hippocampus and cerebral cortex were dissected.

Tissue preparation

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

Cytokines (TNF-α, IL-1β, IL-6) and chemokine CCL2 (MCP-1) assay

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

Nitrite assay

Nitrite levels were measured using the Griess reaction; 100 μL of supernatant of hippocampus and cerebral cortex were mixed with 100 μL Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature. The absorbance was measured on a microplate reader at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards (Green et al. 1982).

Acute-phase protein assay

Acute-phase proteins (CRP and α_1-Acid glycoprotein) in serum were determined by a colorimetric assay with commercially available kits (BioSystems® and Bioclin®, Brazil).

Differential leukocyte count

Cytological slide smears stained with May-Grunwald/Giemsa were used for leukocyte differential counts with a light microscope, and total leukocytes were diluted in Thoma solution (1:20) and counted in a Neubauer chamber by light microscopy (Frode-Saleh et al. 1999).

Protein determination

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

Statistical determination

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

Results

Firstly, we evaluated the effect of acute Hcy administration on cytokine levels (TNF-α, IL-1β and IL-6) and chemokine CCL2 (MCP-1) in the hippocampus of rats. Figure 1 shows that Hcy significantly increased the levels of TNF-α (15 min: [$t(6)=8.17$; $p<0.05$]; 1 h: [$t(6)=3.92$; $p<0.001$], but not at 6 h: [$t(6)=0.67$; $p>0.05$] after acute administration. IL-1β and IL-6 levels also increased 15 min, 1 h and 6 h after Hcy injection, as compared to control (15 min: [$t(6)=5.72$; $p<0.01$]; 1 h: [$t(6)=10.11$; $p<0.01$]; 6 h: [$t(6)=8.49$; $p<0.01$]); and (15 min: [$t(6)=10.30$; $p<0.01$], 1 h: [$t(6)=3.90$; $p<0.05$]; 6 h: [$t(6)=4.71$; $p<0.01$]), respectively. We also observed that MCP-1 levels significantly increased (15 min: [$t(6)=11.17$; $p<0.001$]; 1 h: [$t(6)=8.31$; $p<0.001$] and 6 h: [$t(6)=24.17$; $p<0.001$]) in the hippocampus of rats after Hcy administration. Twelve hours after acute Hcy administration, no alterations in the levels of cytokines and chemokine CCL2 were observed (TNF-α [$t(6)=0.83$; $p>0.05$]; IL-1β [$t(6)=0.36$; $p>0.05$]; IL-6 [$t(6)=1.08$; $p>0.05$] and MCP-1 [$t(6)=0.94$; $p>0.05$]).

We also investigated the effect of acute administration of Hcy on cytokines (TNF-α, IL-1β and IL-6) and chemokine CCL2 (MCP-1) levels in the cerebral cortex of rats. Figure 2 shows that Hcy significantly increased TNF-α (15 min: [$t(6)=4.09$; $p<0.05$]; 1 h: [$t(6)=8.88$; $p<0.001$]; IL-1β (15 min: ([$t(6)=6.35$; $p<0.01$]; 1 h: [$t(6)=5.24$; $p<0.05$] and 6 h: [$t(6)=4.49$; $p<0.05$]), and IL-6 levels (15 min: [$t(6)=5.52$; $p<0.01$]; 1 h: [$t(6)=3.69$; $p<0.05$] and 6 h: [$t(6)=3.24$; $p<0.05$]) after acute administration, when compared to control. We also observed that Hcy administration significantly increased MCP-1 levels (15 min: [$t(6)=7.84$; $p<0.001$]; 1 h: [$t(6)=11.52$; $p<0.001$]). However, at 6 h after Hcy administration, we did not observe any alterations in TNF-α [$t(6)=1.47$; $p>0.05$] and MCP-1 [$t(6)=2.24$; $p>0.05$]. In addition, at 12 h after hyperhomocysteinemia the inflammatory parameters studied were not altered (TNF-α [$t(6)=0.06$; $p>0.05$]; IL-1β [$t(6)=1.08$; $p>0.05$]; IL-6 [$t(6)=0.71$; $p>0.05$] and MCP-1 [$t(6)=0.05$; $p>0.05$]).

Next, we measured the nitrite levels after acute Hcy administration, in hippocampus and cerebral cortex of rats. Figure 3 shows that Hcy significantly increased the nitrite levels in hippocampus and cerebral cortex at 15 min, 1 h and 6 h after Hcy injection, as compared to control (15 min: [$t(6)=5.18$; $p<0.01$]; 1 h: [$t(6)=4.14$; $p<0.05$]; 6 h: [$t(6)=3.65$; $p<0.01$]) and (15 min: [$t(6)=8.43$; $p<0.001$]; 1 h:

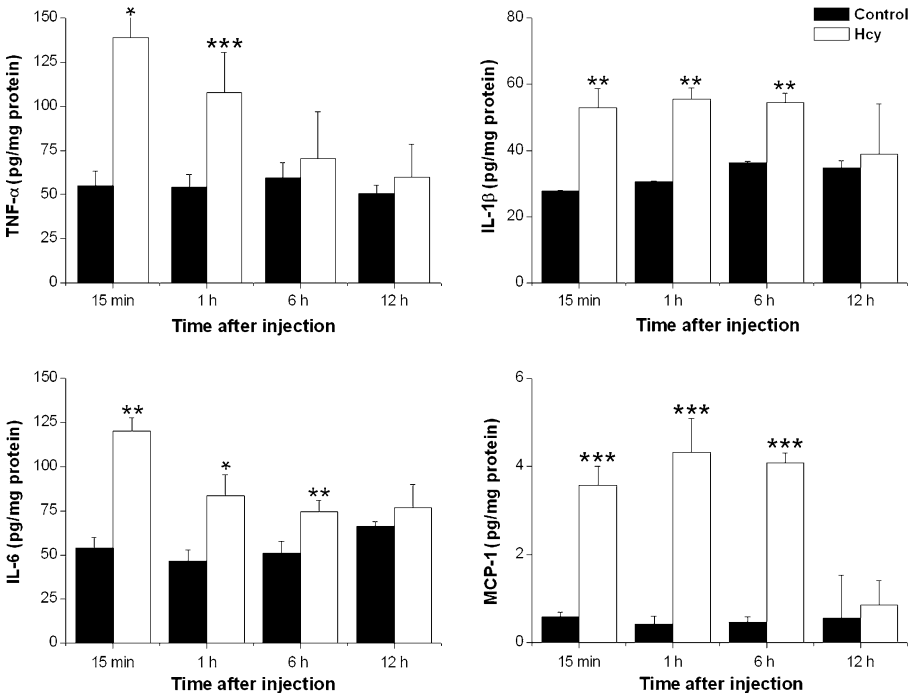

Fig. 1 Effect of acute administration of homocysteine on cytokines (TNF-α, IL-1β, IL-6) and chemokine CCL2 (MCP-1) levels in the hippocampus of rats. Results are expressed as mean±SD for six animals in each group. Different from control, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (Student's *t*-test). Hcy: homocysteine; TNF-α: tumor necrosis factor alpha; IL-1 β: interleukin-1 beta; IL-6: interleukin-6; MCP-1: monocyte chemoattractant protein-1

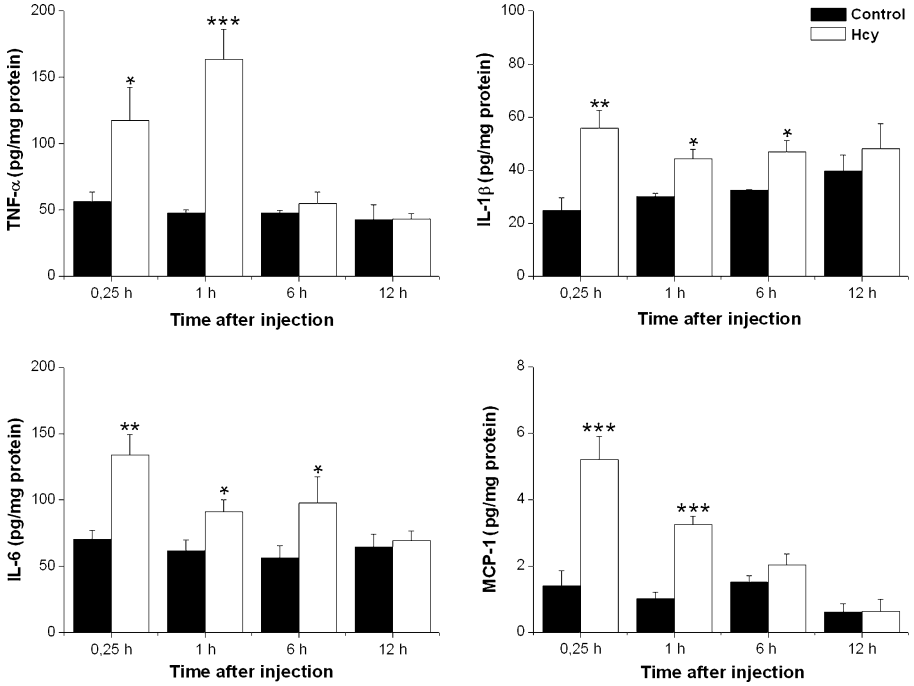

Fig. 2 Effect of acute administration of homocysteine on cytokines (TNF-α, IL-1β, IL-6) and chemokine CCL2 (MCP-1) levels in the cerebral cortex of rats. Results are expressed as mean±SD for six animals in each group. Different from control, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (Student's *t*-test). Hcy: homocysteine; TNF-α: tumor necrosis factor alpha; IL-1 β: interleukin-1 beta; IL-6: interleukin-6; MCP-1: monocyte chemoattractant protein-1

[$t(6)=3.35$; $p<0.05$]; 6 h: [$t(6)=3.82$; $p<0.05$]), respectively. Nitrite levels were not altered in the hippocampus [$t(6)=1.28$; $p>0.05$] and cerebral cortex [$t(6)=0.32$; $p>0.05$] of rats at 12 h after acute hyperhomocysteinemia.

We also evaluated the effect of acute administration of Hcy on cytokine (TNF-α, IL-6) levels in the serum of rats. Figure 4 shows that rats sacrificed at 15 min and 1 h after Hcy injection presented a significant increase in TNF-α levels in the serum, when compared to the control group, [$t(6)=2.76$; $p<0.05$] and [$t(6)=3.82$; $p<0.05$], respectively. However, animals sacrificed at 6 h: [$t(6)=1.26$; $p>0.05$] and 12 h: [$t(6)=0.32$; $p>0.05$] after Hcy administration did not present any alteration in this parameter. In addition, IL-6 levels were increased at 15 min: [$t(6)=5.69$; $p<0.01$], 1 h: [$t(6)=6.34$; $p<0.01$] and 6 h: [$t(6)=10.13$; $p<0.001$], but not at 12 h: [$t(6)=1.29$; $p>0.05$] after Hcy administration.

The effects of acute administration of Hcy on acute-phase proteins (CRP and α_1-Acid glycoprotein) in the serum of rats were also evaluated. Table 1 shows that serum CRP and α1-Acid glycoprotein levels were not altered at any time tested after acute Hcy administration (15 min: [$t(8)=0.14$; $p>0.05$]; 1 h: [$t(8)=0.54$; $p>0.05$]; 6 h: [$t(8)=1.41$; $p>0.05$]; 12 h: [$t(8)=0.11$; $p>0.05$]) and (15 min: [$t(8)=0.28$; $p>0.05$]; 1 h: [$t(8)=1.82$; $p>0.05$]; 6 h: [$t(8)=2.10$; $p>0.05$]; 12 h: [$t(8)=0.04$; $p>0.05$]), respectively.

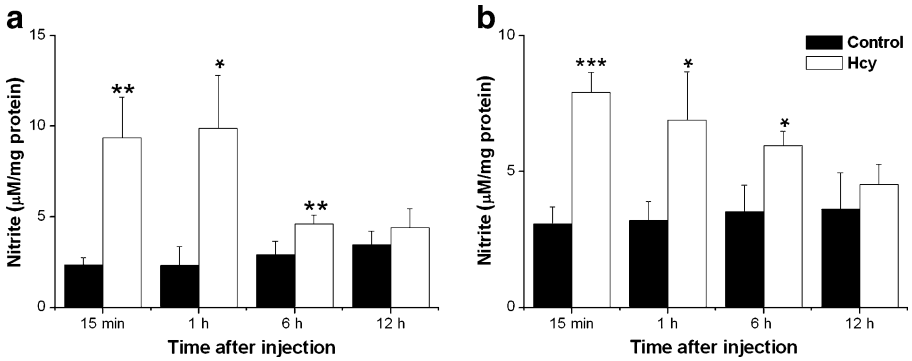

Fig. 3 Effect of acute administration of homocysteine on nitrite levels in the hippocampus (**a**) and cerebral cortex (**b**) of rats. Results are expressed as mean±SD for six animals in each group. Different from control, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (Student's *t*-test). Hcy: homocysteine

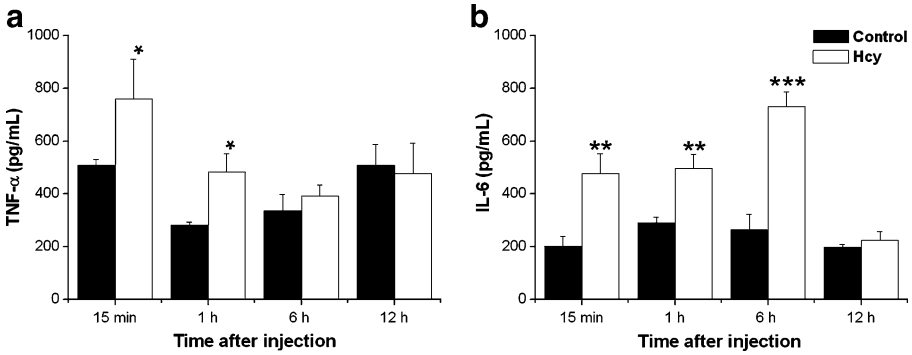

Fig. 4 Effect of acute administration of homocysteine on cytokine (TNF-α, IL-6) levels in the serum of rats. Results are expressed as mean±SD for six animals in each group. Different from control, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (Student's *t*-test). Hcy: homocysteine; TNF-α: tumor necrosis factor alpha; IL-6: interleukin-6

Finally, we investigated the effect of acute administration of Hcy on the differential count of blood leukocytes in rats. Table 2 shows a significant increase in the percentage of cells with a predominance of neutrophils in the blood (15 min: [$t(8)=2.89$; $p<0.01$]; 1 h: [$t(8)=2.27$; $p<0.05$]) and monocytes (15 min: [$t(8)=2.39$; $p<0.05$]; 1 h: [$t(8)=2.48$; $p<0.05$]) after Hcy administration, when compared to the control group. In addition, the percentage of neutrophils and monocytes was not altered after acute hyperhomocysteinemia (6 h: [$t(8)=0.22$; $p>0.05$]; 12 h: [$t(8)=1.97$; $p>0.05$]) and (6 h: [$t(8)=0.49$; $p>0.05$] and 12 h: [$t(8)=0.82$; $p>0.05$]), respectively. Nevertheless, the total number of leukocytes did not change significantly after Hcy administration, as compared to the control group (data not shown).

Discussion

Homocystinuria is an inborn error of metabolism, characterized by a severe deficiency of cystathionine β-synthase activity. Affected patients present tissue accumulation of Hcy and a variable symptomatology, including mental retardation, epilepsy, seizures and atherosclerosis, whose pathophysiology is poorly understood (Mudd et al. 2001).

Since Hcy and inflammatory parameters seem to be associated with the pathogenesis of several diseases (Gori et al. 2005; Weiss et al. 2003; Welch and Loscalzo 1998), in the present study, we evaluated the effects of acute hyperhomocysteinemia on important markers of inflammation such as cytokines, chemokine CCL2, nitrite levels and acute-phase proteins in hippocampus, cerebral cortex and serum of rats. In addition, in order to evaluate the blood leucocyte profile, differential counts of leukocytes were also performed. Firstly, we investigated the effect of Hcy administration on the hippocampus and cerebral cortex of rats. Results showed that Hcy increases the cytokines IL-1β and IL-6, chemokine CCL2 (MCP-1) and nitrite levels in both cerebral structures studied from rats sacrificed at 15 min, 1 h and 6 h, whereas TNF-α was increased only at 15 min and 1 h after acute administration. However, 12 h after acute administration of Hcy, onwards, there was no effect on cytokines and chemokine CCL2. It is possible that the increase in the levels of TNF-α, IL-1β, IL-6, MCP-1 and nitrite probably depend on Hcy, since a previous study showed that this amino acid presents a peak in the brain at 15 min after injection, returning to baseline levels after 12 h (Streck et al. 2002)

It has been reported that proinflammatory cytokines and other mediators play an essential role in CNS inflammation

Table 1 Effect of acute administration of homocysteine on acute-phase protein (CRP, α_1-Acid glycoprotein) levels in the serum of rats

Group	CRP (mg/L)	α_1-Acid glycoprotein (mg/dL)
Control 15 min	0.14±0.10	12.2±3.11
Hcy 15 min	0.13±0.73	11.60±3.01
Control 1 h	0.25±0.14	17.80±5.49
Hcy 1 h	0.20±0.09	12.70±4.43
Control 6 h	0.11±0.05	14.20±1.30
Hcy 6 h	0.19±0.08	10.50±3.64
Control 12 h	0.17±0.04	13.50±2.88
Hcy 12 h	0.18±0.10	13.42±2.50

Results are expressed as mean±SD for eight animals in each group (Student's *t*-test)

Hcy homocysteine, *CRP* C-reactive protein

Table 2 Effect of acute administration of homocysteine on the differential count of leukocytes (% of leukocytes) in blood of rats

Group	Neutrophils %	Lynfocytes %	Monocytes %
Control 15 min	17.50±4.23	80.33±4.08	2.00±0.63
Hcy 15 min	30.50±10.15**	68.00±9.87*	3.35±1.20*
Control 1 h	22.17±2.98	75.50±3.14	2.33±0.51
Hcy 1 h	29.00±6.73*	66.50±8.50*	4.50±2.07*
Control 6 h	22.50±5.84	74.33±6.37	3.17±0.75
Hcy 6 h	23.50±9.26	73.67±10.15	2.83±1.47
Control 12 h	21.80±2.86	74.80±3.03	3.20±1.09
Hcy 12 h	26.20±4.08	69.80±5.71	4.00±1.87

Results are expressed as mean±SD for eight animals in each group (Student's *t*-test)

Hcy homocysteine

(Rothwell and Luheshi 2000). Activated microglia may secrete a diverse range of proinflammatory cytokines and neurotoxic factors, such as NO and reactive species of oxygen (ROS), which contribute to neuronal damage in neurodegenerative diseases (Liu and Hong 2003; Lerouet et al. 2002). In this context, hyperhomocysteinemia has been demonstrated to induce neuronal death, often associated with increased levels of ROS formation (Lipton et al. 1997). Indeed, we have previously demonstrated that chronic Hcy treatment induces oxidative stress in the cerebrum of rats, increasing lipid peroxidation and reducing enzymatic and non-enzymatic antioxidant defenses (Matté et al. 2007, 2009; Streck et al. 2003). Therefore, these findings may be closely related to the increase in proinflammatory cytokines and nitrite levels elicited by Hcy in brain, observed in the present study, since proinflammatory cytokines are often produced in response to oxidative stress and, conversely, may act to cause oxidative stress in their target cells (Halliwell and Gutteridge 2007).

Other reports are in agreement with our data showing a relationship between hyperhomocysteinemia and proinflammatory state (Gori et al. 2005; de Jong et al. 1997). Some authors have shown that Hcy treatment stimulates MCP-1 expression in several cell types (Sung et al. 2001, Wang et al. 2000, 2001). In addition, the pre-treatment of cells with nuclear factor Kappa β (NF-κβ) inhibitors can alleviate the stimulatory effect of Hcy on MCP-1 expression, supporting the notion that Hcy-stimulated chemokine expression is mediated via NF-κB activation (Wang et al. 2000, 2001; Au-Yeung et al. 2003). In turn, NF-κB plays a key role in the orchestration of inflammatory and immune responses by controlling transcription of genes encoding adhesion molecules and cytokines (Yun et al. 2009; Zhou et al. 2007; Dalal et al. 2003).

A number of studies have indicated that Hcy may contribute to the progression of atherosclerosis, in part by enhancing vascular inflammation (Sung et al. 2001; Wang et al. 2000, 2001). Hcy has been shown to promote a potent stimulation of IL-6 expression in cultured rat aorta vascular smooth muscle cells (VSMCs) (Zhang et al. 2006). In vitro studies have shown that Hcy is able to induce mRNA and protein expression of the proinflammatory cytokines, IL-8 and MCP-1 in cultured human aortic endothelial cells (HAECs) (Poddar et al. 2001; Sung et al. 2001). In contrast, it had no effect on the expression of other cytokines, such as TNF-α, IL-1β, granulocyte/macrophage colony-stimulating factor (GM-CSF) and transforming growth factor-β (TGF-β) (Poddar et al. 2001).

In order to verify the systemic effects of Hcy on inflammation, we also measured the levels of TNF-α and IL-6 in the serum of rats submitted to acute hyperhomocysteinemia. Results revealed that Hcy significantly increases these cytokines, where IL-6 levels increased at 15 min, 1 h and 6 h, whereas TNF-α was increased only at 15 min and 1 h after administration. Similarly to effects observed in the brain, we did not observe any alteration at 12 h after Hcy administration.

It has been shown that Hcy may promote endothelial dysfunction, probably associated with oxidative stress, which can lead to the activation of proinflammatory pathways in the vasculature (Weiss 2005; Zhang et al. 2001; Liu et al. 2008). TNF-α is one of the central mediators of tissue inflammation and induces synthesis of other inflammatory cytokines (Lucas et al. 2006), whilst elevated IL-6 levels are closely related to an increased risk of myocardial infarction (Lindmark et al. 2001; Ridker et al. 2000a); as such, our findings that demonstrate that Hcy increases both TNF-α and IL-6 levels in serum of rats suggest an important role of Hcy in the induction of a systemic inflammatory response.

On the other hand, the local release of TNF-α can lead activate neutrophils and endothelial cells to further upregulate the expression of adhesion molecules so that activated neutrophils can bind and migrate across the endothelial cell barrier (Argenbright and Barton 1992). Moreover, it has been demonstrated that Hcy in human can enhance the adhesion of leukocytes to the vascular endothelium and lead to leukocyte-mediated changes in endothelial integrity and function, ultimately resulting in thromboses and vascular lesion (Dudman et al. 1999). Together, these events facilitate the initiation and progression of atherosclerosis lesion (Weiss 2005; Napoli et al. 2001) and might explain why mild hyperhomocysteinemia has been described as an important risk factor for neurodegenerative and vascular diseases (Mattson and Shea 2003).

Acute-phase proteins are produced by the liver in large quantities during an inflammatory state (Patti et al. 2002). Furthermore, it has been suggested that acute-phase proteins have an essential role in the inhibition of extracellular proteases, blood clotting, fibrinolysis and modulation of immune cell function (Ridker et al. 2000b). Considering that we have previously reported that histological analysis reveals the presence of inflammatory infiltrate in liver tissue sections from hyperhomocysteinemic rats (Matté et al. 2009); in this study, acute-phase proteins, CRP and α1-Acid glycoprotein, were also evaluated. However, results showed that Hcy did not alter these parameters at any time tested. These data are in agreement with clinical findings from other investigators who did not observe alterations in the CRP levels in older subjects with hyperhomocysteinemia (Gori et al. 2005).

Regarding the blood leucocyte profile, the analysis of differential counts of leukocytes showed an increase in the relative number of neutrophils and monocytes at 15 min and 1 h, but not at 6 h or 12 h, after Hcy administration. However, the total number of leukocytes did not change

(data not shown). It has been reported that cytokines can induce neutrophilic inflammation (Jatakanon et al. 1999); on the other hand, neutrophils can also be important sources of cytokines such as TNF-α (Thomas et al. 1995). Interestingly, we found that Hcy increased TNF-α levels at the same times point that neutrophilia was observed (15 min and 1 h after Hcy injection), suggesting a possible relationship between TNF-α and the increase in neutrophils. However, further studies are needed to determine the mechanism of neutrophilia and monocytosis elicited by Hcy.

In summary, in the present study, we demonstrated that acute Hcy administration induces immune activation by increasing cytokines, chemokine and nitrite levels in the hippocampus, cerebral cortex and serum of rats, in addition to increasing the relative number of neutrophils and monocytes in the blood. Our findings provide insights into the role of Hcy in the pathogenesis of human vascular inflammation, cerebrovascular and neurodegenerative disease and facilitate the identification of new therapeutic approaches in the treatment of homocystinuric patients. However, many questions and cellular mechanisms by which hyperhomocysteinemia exert these effects remain to be answered.

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Capítulo II

Neurochemical Research

CHRONIC HYPERHOMOCYSTEINEMIA INCREASES INFLAMMATORY MARKERS IN HIPPOCAMPUS AND SERUM OF RATS

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CHRONIC HYPERHOMOCYSTEINEMIA INCREASES INFLAMMATORY MARKERS IN HIPPOCAMPUS AND SERUM OF RATS

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Abstract

This study investigated the effects of chronic homocysteine administration on some parameters of inflammation, such as cytokines (TNF-α, IL-1β and IL-6), chemokine CCL_2 (MCP-1), nitrite and prostaglandin E_2 levels, as well as on immunocontent of NF-κB/p65 subunit in hippocampus and/or serum of rats. Based on the fact that acetylcholinesterase promotes inflammation, we also evaluated the effect of homocysteine on this enzyme in hippocampus of rats. Wistar rats received daily subcutaneous injections of homocysteine (0.3-0.6 μmol/g body weight) or saline (control) from the 6^{th} to the 28^{th} days-of-age. One or 12 h after the last injection, rats were euthanized and hippocampus and serum were used. Results showed that chronic hyperhomocysteinemia significantly increased pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), chemokine CCL_2 (MCP-1) and prostaglandin E_2 at 1 and 12 h after injection of this amino acid in hippocampus and/or serum of rats. Nitrite levels increased in hippocampus, but decreased in serum at 1 h after chronic hyperhomocysteinemia. Acetylcholinesterase activity and immunocontent of citoplasmic and nuclear NF-κB/p65 subunit were increased in hippocampus of rats subjected to hyperhomocysteinemia 1 h after administration, but did not alter at 12 h. According to our results, chronic hyperhomocysteinemia increases inflammatory parameters, suggesting that this process might be associated, at least in part, with the cerebrovascular and vascular dysfunctions characteristic of some homocystinuric patients.

Keywords: Hyperhomocysteinemia; Cytokines; Nitrite levels; Prostaglandin E_2; NF-κB/p65 subunit; Acetylcholinesterase activity.

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 [1]. Hyperhomocysteinemia also occurs in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases [2-4], and has been identified as an independent risk factor for atherosclerosis, cerebrovascular and neuroinflammatory diseases [5-9].

Inflammation is fundamentally a protective response whose ultimate goal is to eliminate the injury-inducing agent, including a micro-organism, physical stimuli and chemical agent [10]. Some of the important mediators of inflammation are: histamine, serotonin, lysosomal enzymes, prostaglandins (PGs), leukotrienes (LTs), platelet activating factor, reactive oxygen species (ROS), nitric oxide (NO), cytokines, chemokines, acute-phase proteins, the coagulation/fibrinolysis system, and the complement system [10]. Exposure of the cells to diverse stimuli, such as inflammatory cytokines, oxidative stress, or bacterial endotoxins, results in activation of nuclear factor-kappaB (NF-κB) through the stimulation of phosphorylation and degradation of IκBα [11].

Activated NF-κB is then translocated to the nucleus, where it binds to the cis-acting κB enhancer element of target genes and activates the expression of pro-inflammatory mediators [12]. NF-κB plays an important role in inflammatory phenotypic changes in various pathophysiological conditions [13]. Like other members of the NF-κB family, NF-κB/p65 resides in the cytoplasm in an inactive form bound to inhibitory IκB proteins. Cellular activation results in the nuclear translocation of NF-κB/p65 for initiating gene transcription. The translocation of NF-κB/p65 from cytoplasm to nuclear is often taken as an indication of NF-κB

activation and is related to the cellular response to oxidants or to the inflammatory and acute immune response [14].

Brain inflammation is characterized by activation of microglia and astrocytes, expression of key inflammatory mediators, but limited invasion of circulating immune cells [15]. Inflammation induces rapid expression of key inflammatory mediators, which in turn, up-regulate adhesion molecules, increase permeability of the blood-brain barrier, facilitate the invasion of peripheral immune cells, induce the release of potentially toxic molecules and compromise brain cells [16]. Among the diverse functions that are regulated by acetylcholinesterase (AChE), inflammation has recently emerged as one of the most interesting. Since, acetylcholine (Ach) is a neurotransmitter and has regulatory effect on serotonin, dopamine and other neuropeptides, it is clear that a complex network of interaction exists between these molecule in the regulation of immune response and neurotransmission [17,18].

The relative balance of pro and anti-inflammatory cytokine and chemokine expression is believed to play a significant role in the etiology of both thrombosis and atherogenesis [19]. Previous studies have indicated that Hcy may contribute to the development and progression of atherosclerosis by inducing endothelial dysfunction, increasing proliferation of vascular smooth muscle cell, promoting lipoprotein oxidation and platelet activation, and enhancing collagen synthesis [20]. In addition, a pro-inflammatory state, associated with hyperhomocysteinemia, has been demonstrated by several authors [7, 21,22]. In vitro studies have shown that Hcy is able to induce mRNA and protein expression of the pro-inflammatory cytokines in cultured human aortic endothelial cells [23,24]. Additionally, we have previously reported that

acute Hcy administration increases pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and chemokine CCL_2 (MCP-1) in brain and serum of rats [9].

In order to investigate whether chronic hyperhomocysteinemia could alter inflammatory markers, in the present study we evaluated the effect of chronic Hcy administration on some inflammatory parameters such as cytokines (TNF-α, IL-1β and IL-6), chemokine CCL_2 (MCP-1), nitrite and prostaglandin E_2 levels and the immunocontent of NFκB/p65 subunit, as well as on acetylcholinesterase activity in hippocampus and/or serum of rats.

Materials and Methods

Animals and reagents

Seventy-four Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12h light/12h dark cycle at a constant temperature (22 ± 1 ^{o}C), with free access to water and commercial protein chow. Animal care followed the NIH ‘‘Guide for the Care and Use of Laboratory Animals’’ (NIH publication no. 80-23, revised 1996) and was approved by the University Ethics Committee.

Acrylamide, bisacrylamide, SDS, and β-mercaptoethanol used in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit IgG peroxidase-conjugated and reagents to detect chemioluminescence (ECL) were purchased from Amersham Pharmacia

Biotech (Piscataway, NJ, USA). Hybond-C nitrocellulose membranes were from Hybond-ECL-(Hybond- ECL- nitrocellulose membrane, Amersham Biosciences, Freiburg, Germany). X-ray films were purchased from Kodak (Kodak X-Omat, Rochester, NY, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Chronic homocysteine treatment

D,L-Hcy was dissolved in 0.9% NaCl solution (saline) and buffered to pH 7.4. Hcy solution was administered subcutaneously twice a day from the 6^{th} to 28^{th} days-of-age. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory [25]. 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 reached levels similar to those found in homocystinuric patients [1,25]. Hcy reaches the brain maximum concentration between 15 and 30 min after injection and returns to baseline levels at 12 h [25]. Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. The rats were euthanized by decapitation without anesthesia 1 h or 12 h after the last injection; serum was separated and brain was quickly removed and hippocampus was dissected.

Western blotting for cytosolic and nuclear NF-κB/p65 subunit

Tissue hippocampus were homogenized in 300 µL hypotonic lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM

phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM NaF, 1 mM sodium orthovanadate plus protease inhibitor cocktail). Hippocampus homogenate were than lysed with 18 µL 10% IGEPAL. The homogenate was centrifuged (14000 x g, 30 s, 4^{o}C), and supernatants containing the cytosolic fraction were stored at -80^{o}C. The nuclear pellet was resuspended in 200 µL ice-cold hypertonic extraction buffer (10 mM HEPES (pH 7.9), 0.40 M NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM NaF, 1 mM sodium orthovanadate, 0,25 mM EDTA, 25% glycerol plus protease inhibitor cocktail). After 40 min of intermittent mixing, extracts were centrifuged (14000 x g, 10 min, 4^{o}C), and supernatants containing nuclear protein were secured [26]. Cytosolic and nuclear fractions were used for NF-κβ/p65 subunit Western blotting. Aliquots were taken for protein determination and, for electrophoresis analysis, were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris-HCl, pH 6.8. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). Protein samples were separated by 10% SDS-PAGE (50µg/lane of total protein) and transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15V in transfer buffer (48 mMTrizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% bovine serum albumin (BSA)). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05% Tween-20 (T-TBS) and then incubated overnight at 4ºC in blocking solution containing one of the following antibodies: anti-NF-kB/p65 (1:1000;

Santa Cruz Biotechnology) and anti-β-actin (1:1000, Sigma Chemical Co.). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:1000. The blot was again washed twice for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). The chemioluminescence was detected using X-ray films that were scanned and analyzed using the Optiquant Software (Packard Instruments).

Tissue preparation

For acquisition of serum, whole blood was centrifuged at 1000 x g for 5 min and the serum was immediately removed. Hippocampus was homogenized 1:5 (w/v) in saline solution (0.9% NaCl). The homogenate was centrifuged at 800 x g for 10 min at 4^{o}C and the supernatant was used in assays.

Cytokine (TNF-α, IL-1β and IL-6) and chemokine CCL_2 (MCP-1) assay

TNF-α, IL-1β, IL-6 and MCP-1 levels in hippocampus and serum were quantified by Multiplexed Immunoassay with a commercially available kit, and analyzed on a Luminex 200$^{®}$TM.

Nitrite assay

Nitrite levels were measured using the Griess reaction; 100 µL of rat hippocampus supernatant or serum were mixed with 100 µL Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well

plates for 10 min at room temperature. The absorbance was measured on a microplate reader (SpectraMax M5/M5 Microplate Reader, Molecular Devices, MDS Analytical Technologies, Sunnyvale, California, USA) at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards [27].

Prostaglandin E_2 assay

PGE_2 was measured by the method described by Wallace et al. (1988) and determined by radioimmunoassay [28].

Acetylcholinesterase activity

AChE activity was determined, according to Ellman et al. (1961), with some modifications. Hydrolysis rates were measured at an ACh concentration of 0.8 mM in 1 mL assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB at 25^{o}C. About 50 µL of rat hippocampus supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2-3 min (intervals of 30 s) [29].

Protein determination

Protein concentrations were measured by the method of Lowry et al. (1951) or Bradford (1976) using bovine serum albumin as standard [30, 31].

Statistical determination

Data were analyzed by the Student`s t test for unpaired samples. All analyses were performed using the Statistical Package for the Social Sciences

(SPSS) software with a PC-compatible computer. Differences were considered statistically significant if $p < 0.05$.

Results

We first studied the effect of chronic Hcy administration on cytokine levels (TNF-α, IL-1β and IL-6) and chemokine CCL_2 (MCP-1) in hippocampus of rats. Fig. 1A shows that Hcy significantly increased the levels of TNF-α at 1 h [$t(7) = 4.15$; $p < 0.01$], but not at 12 h [$t(7) = 0.80$; $p > 0.05$] after chronic hyperhomocysteinemia. In addition, IL-1β and IL-6 levels were altered. Fig 1B shows that IL-1β was increased at 1 h [$t(7) = 5.47$; $p < 0.001$] and at 12 h [$t(7) = 3.65$; $p < 0.01$] after Hcy injection, as compared to the control group. Similarly, IL-6 was increased at 1 h [$t(7) = 4.30$; $p< 0.01$] and at 12 h [$t(7) = 3.29$; $p < 0.05$] after the last administration of Hcy (Fig 1C). Besides, we also observed that MCP-1 levels significantly increased at 1 h [$t(7) = 3.55$; $p < 0.01$], but not at 12 h [$t(7) = 1.34$; $p > 0.05$], after chronic hyperhomocysteinemia (Fig 1D).

The effect of chronic hyperhomocysteinemia on cytokines (TNF-α, IL-1β and IL-6) and chemokine CCL_2 (MCP-1) levels was also investigated in serum of rats. Fig. 2A shows that Hcy significantly increased TNF-α levels at 1 h [$t(8) = 8.58$; $p < 0.001$] and at 12 h [$t(7) = 2.92$; $p < 0.05$] after the last injection of this amino acid. Fig 2B shows that IL-1β was increased at 1 h [$t(8) = 5.43$; $p< 0.001$] and at 12 h [$t(8) = 4.24$; $p<0.01$] after Hcy injection. Similarly, chronic hyperhomocysteinemia significantly increased IL-6 levels at 1 h [$t(8) = 10.58$; $p < 0.001$] and at 12 h [$t(8) = 4.43$; $p < 0.01$] (Fig 2C); and MCP-1 levels at 1 h [$t(8) = 6.11$; $p < 0.001$] and at 12 h [$t(8) = 3.56$; $p < 0.01$] (Fig 2D), as compared to the control group.

Next, the nitrite levels were determined in hippocampus and serum of animals subjected to chronic Hcy administration. Fig. 3A shows that Hcy significantly increased the nitrite levels in hippocampus at 1 h [$t(7) = 2.98$; $p < 0.05$] and at 12 h [$t(6) = 2.63$; $p < 0.05$], as compared to the control group. On the other hand, Fig 3B shows that Hcy significantly decreased the nitrite levels in serum at 1 h [$t(8) = 2.32$; $p < 0.05$], but did not alter this parameter at 12 h [$t(6) = 0.91$; $p > 0.05$] after chronic administration of this amino acid.

Subsequently, the effect of chronic Hcy administration on prostaglandin E_2 was evaluated in the hippocampus and serum of rats. Fig. 4A shows that rats euthanized at 1 h [$t(6) = 5.95$; $p < 0.001$] and at 12 h [$t(6) = 10.25$; $p < 0.001$] after chronic hyperhomocysteinemia presented a significant increase in prostaglandin E_2, as compared to the control group. In addition, Fig 4B shows that Hcy injection increased prostaglandin E_2 levels in serum of rats at 1 h [$t(6) = 2.94$; $p < 0.05$]; however, animals euthanized at 12 h [$t(6) = 1.08$; $p> 0.05$] after chronic hyperhomocysteinemia did not present alterations in this parameter.

Since, NF-κB regulate innate immune response, and it is activated rapidly in response to a wide range of stimuli, including pro-inflammatory cytokines, such as TNF-α and IL-1β [32], we investigated the effect of chronic hyperhomocysteinemia on immunocontent of cytosolic and nuclear fraction of NF-κB/p65 subunit. Fig. 5A shows that chronic Hcy administration significantly increased the immunocontent of cytosolic [$t(7) = 5.36$; $p<0.001$] and nuclear fraction of NF-kB/p65 subunit [$t(8) = 3.15$; $p<0.01$] in the hippocampus of rats at 1 h after the last injection of this amino acid. Fig. 5B shows that chronic hyperhomocysteinemia had no effect on immunocontent of cytosolic [$t(7) =$

0.242; p>0.05] and nuclear NF-kB/p65 subunit [t(5) = 0.007; p>0.05] at 12 h after hyperhomocysteinemia.

Considering that AChE is an important regulator of the inflammatory process; we determined the effect of chronic Hcy administration on activity of AChE. Fig. 6 shows that chronic hyperhomocysteinemia provoked an increase in the AChE activity in the hippocampus of rats at 1 h [t(6) = 3.15; p < 0.05], but did not alter this enzyme at 12 h [t(7) = 0.53; p> 0.05] after the last injection.

Discussion

Hyperhomocysteinemia has been associated with vasculopathy in the peripheral and cerebral blood vessels [33,34]. The mechanism by which hyperhomocysteinemia promotes endothelial dysfunction and subsequent vascular disease has recently been explored in the peripheral vessel system, but less extensively in the cerebral blood vessels [35-37].

Previous work using culture models has suggested possible cytokine involvement in the pathogenesis of hyperhomocysteinemia [24,38,39], in the present study we initially investigated the effect of chronic hyperhomocysteinemia on cytokines in hippocampus and serum of rats.Results showed that chronic Hcy administration increases cytokines IL-1β and IL-6 in hippocampus of rats at 1 or 12 h after the last injection of this amino acid, whereas TNF-α and MCP-1 were increased only at 1 h after chronic hyperhomocysteinemia. Although the precise mechanisms of Hcy action on the inflammatory process are not fully understood, our findings suggest that the increase in cytokine levels could be closely related to the high brain and plasma levels of this amino acid, which achieve the peak as soon at 15 min after

injection, returning to baseline levels after 12 h [25]. These data are in agreement with Su et al. (2005), who reported that Hcy in vitro induces mRNA and protein expressions of the inflammatory cytokines TNF-α, IL-1β, IL-6, IL-8, and IL-12 [39]. Most importantly, there is now extensive evidence that neuroinflammation contributes to many acute and chronic degenerative disorders and, perhaps, some psychiatric diseases [16], and it is also possible that the cytokines profiles in severe hyperhomocysteinemia described here could contribute to the cognitive impairment that is frequently observed in this disease.

Although several central nervous system (CNS) cell types, including microglia, astrocytes and neurons, are able to secrete cytokines, studies support the involvement of peripherally-derived cells in contributing to brain inflammation and injury [40]. It has also been suggested that an associated blood-brain barrier dysfunction may occur whereby a leaky state promotes transendothelial migration of immune cells [40]. With regard to cytokines that are important modulators of inflammatory events [41], we have previously shown that acute Hcy administration increases the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and MCP-1 in brain and serum of rats [9].

In the present study, chronic Hcy administration increased TNF-α, IL-1β, IL-6 and MCP-1 in rat serum at 1 and 12 h after the last injection of this amino acid. It has recently been shown that Hcy may contribute to the progression of atherosclerosis, in part by enhancing vascular inflammation [23,42]. Following Hcy-induced injury, endothelial cells are activated and are capable of producing various adhesion molecules and chemokines such as, VCAM-1, ICAM-1, E-selectin, P-selectin, β1-integrin, IL-8 which participate in inflammatory reactions

in the arterial wall [24,43-46]. Other potentially important inflammatory actions of Hcy include stimulation of TNF-α release by blood monocytes and increased adherence of human monocytes to endothelial cells [38, 39]. These results suggest that Hcy may contribute to the initiation and progression of vascular disease by promoting monocyte activation, resulting in the secretion of cytokines that might amplify the inflammatory response in the arterial wall.

Moreover, we also evaluated the effect of chronic hyperhomocysteinemia on nitrite levels. Hcy significantly increased nitrite levels at 1 h and 12 h in hippocampus of rats after the Hcy injection. NO is produced by a group of enzymes called neuronal nitric oxide synthase (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). These enzymes convert arginine into citrulline, producing NO in the process [47-49]. The activity of the NOS enzymes is subject to discreet and multiple interconnected mechanisms of regulation. NO regulates a diverse range of physiological and cellular processes, including endothelial cell migration, proliferation, extracellular matrix degradation, platelet function, angiogenesis and mitogenesis, which are all crucial for cardiovascular physiology [50,51]. During inflammation, NO levels increase considerable, due to the induction of iNOS by cytokines [52]. In this context, Welch et al. (1998) reported that Hcy induced NO synthesis in the vascular smooth muscle cells after NFκβ-dependent transcriptional activation of iNOS [53]. In accordance with our results, it has been reported that acute Hcy administration increases nitrite levels in rat brain [9].

Contrast data were obtained for rat serum; Hcy decreased nitrite levels at 1 h after chronic hyperhomocysteinemia, but did not alter this parameter at 12 h. Under normal conditions, NO plays a role in the detoxification of Hcy through

the formation of S-nitrosohomocysteine [54]. However, chronic exposure to Hcy increases the formation of superoxide anion (O_2^-), which can react with NO to yield the potent oxidant, peroxynitrite ($ONOO^-$) [55,56]. In our study, nitrite levels was decreased in serum after chronic hyperhomocysteinemia, and we proposed that the bioavailability of NO may be reduced due to the generation of free radicals and lipid peroxidation caused by Hcy. In this context, Jiang et al. (2005) showed that eNOS activity was significantly reduced by Hcy in endothelial cells of CBS null mice [57]. Further research found that increased vascular oxidant stress in hyperhomocysteinemia not only leads to a decreased NO bioavailability, but also activates redox-sensitive signaling pathways that induce a pro-inflammatory state in the vessel wall [58,59].

Prostaglandins are members of the eicosanoid family and are not stored by cells; rather, they are synthesized from arachidonic acid via the actions of cyclooxygenase enzymes (COX), either constitutively or in response to cell-specific trauma, stimuli, or signaling molecules [60,61]. The most abundant prostanoid in the human body is PGE_2 [62], and has been considered the principal prostaglandin in acute inflammation, as well as in arthritic diseases such as rheumatoid arthritis [63] and osteoarthritis [64]. The effect of chronic Hcy administration on PGE_2 in hippocampus and serum of rats was next investigated. Results showed that chronic Hcy administration increased PGE_2 in the hippocampus at 1 and 12 h after injection. On the other hand, in serum we verified that Hcy increased PGE_2 at 1 h after Hcy administration. It has also been reported that iNOS specifically binds to COX-2 and S-nitrosylates the enzyme on Cys526, resulting in an increased COX-2 catalytic activity and enhanced PGE_2 production [65]. These effects of PGE_2 in hippocampus could

be responsible, at least in part, for the increase in the nitrite levels observed in our study.

Additionally, we evaluated the immunocontent of cytoplamic and nuclear NF-κB/p65 subunit in hippocampus of rats subjected to chronic Hcy administration. We demonstrated that chronic hyperhomocysteinemia significantly increased the immunocontent of cytoplamic and nuclear NF-κB/p65 subunit at 1 h, but did not observe any alteration in the immunocontent of NF-κB/p65 subunit at 12 h after chronic administration of this amino acid. Increased vascular oxidative stress in hyperhomocysteinemia has been shown to activate pro-inflammatory signaling pathways in endothelial cells, including the NF-κβ pathway [66]. In this context, Hcy has been shown to stimulate ICAM-1 and TNF-α expression in endothelial cells, mediated by the activation of NF-κβ, via a mitogen-activated protein kinase (MAPK) pathway [67]. NF-κβ may also be activated though a protein kinase C signaling mechanism, which seems to be stimulated by Hcy [42]. In addition, reports showed that Hcy-induced IL-6 gene expression occurs through the activation of NF-κβ [68]. Furthermore, recent studies have demonstrated the involvement of Hcy actions linked to oxidative stress [69,70], and which NF-κβ is a transcriptional factor whose activation by signaling pathways is correlated with elevated ROS levels [71]. In this context, Matté et al. (2009) showed that chronic Hcy administration increased DNA damage, as evaluated by the comet assay, and disrupted antioxidant defenses (enzymatic and non-enzymatic) in parietal cortex and blood/plasma [69]. It has been previously demonstrated that acute Hcy administration decreases catalase activity (CAT) in rat hippocampus and that vitamins E and C completely prevent this effect, indicating that the participation of oxidative stress

is probably involved in the actions of Hcy [72]. In fact, NF-κβ may be a point of convergence by which different agents cause inflammatory activation in hyperhomocysteinemia.

Acetylcholine is rapidly hydrolyzed by AChE in neural synapses and the motor endplate. Considering the inflammatory suppressive effect of acetylcholine, it is conceivable that AChE activity is an intrinsic regulator of inflammation [73]. Indeed, peritoneal injection of AChE inhibitors reduce serum pro-inflammatory cytokine levels and improve survival in a murine model of sepsis [74]; intravenous AChE inhibitors reduce IL-1β in brain and blood and decrease serum AChE activity in mice [75]; and basal AChE activity in the circulation is inversely related to serum IL-6 levels induced by endotoxin in humans [76]. Based on these data, we also investigated the effect of chronic Hcy administration on AChE activity. We observed that the activity of this enzyme was increased in hippocampus at 1 h after the last injection of Hcy. Considering the role of acetylcholine in inhibiting the release of pro-inflammatory cytokines [73,75], we might speculate that this increase in AChE activity may cause an impairment of acetylcholine ability in regulating the inflammatory processes, which would explain, at least in part, the alterations in cytokine levels described above.

In summary, we showed that chronic hyperhomocysteinemia induced immune activation by increasing cytokines (TNF-α, IL-1β, IL-6), chemokine CCL_2 (MCP-1), nitrite and PGE_2 levels, immunocontent of NF-κB/p65 subunit and AChE activity. Collectively, our results provide an additional insight into the inflammatory mechanisms of Hcy, and may contribute to explain the complex

factors involved in the cerebrovascular and vascular dysfunction exhibited by hyperhomocysteinemic patients.

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Legends to Figures

Figure 1. Effect of chronic administration of homocysteine on cytokine (TNF-α, IL-1β, IL-6) and chemokine CCL_2 (MCP-1) levels in the hippocampus of rats. Results are expressed as mean ± SD for six animals per group. Different from control, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (Student's t-test). Hcy: homocysteine; TNF-α: tumor necrosis factor alpha; IL-1 β: interleukin-1 beta; IL-6: interleukin-6; MCP-1: monocyte chemoattractant protein-1.

Figure 2. Effect of chronic administration of homocysteine on cytokine (TNF-α, IL-1β, IL-6) and chemokine CCL_2 (MCP-1) levels in the serum of rats. Results are expressed as mean ± SD for six animals per group. Different from control, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (Student's t-test). Hcy: homocysteine; TNF-α: tumor necrosis factor alpha; IL-1 β: interleukin-1 beta; IL-6: interleukin-6; MCP-1: monocyte chemoattractant protein-1.

Figure 3. Effect of chronic administration of homocysteine on nitrite levels in the hippocampus (A) and serum (B) of rats. Results are expressed as mean ± SD for six animals per group. Different from control, $^{*}p<0.05$ (Student's t-test). Hcy: homocysteine.

Figure 4. Effect of chronic administration of homocysteine on prostaglandin E_2 in the hippocampus (A) and serum (B) of rats. Results are expressed as mean ± SD for six animals per group. Different from control, $^{*}p<0.05$; $^{***}p<0.001$ (Student's t-test). Hcy: homocysteine.

Figure 5. Effect of chronic hyperhomocysteinemia on cytosolic and nuclear immunocontent of NF-κB/p65 subunit at 1 h (A) and at 12 h (B) after homocysteine administration in hippocampus of rats. Bars represent the mean ± SD for 4-6 animals in each group. Different from control, $^{**}p<0.01$; $^{***}p<0.001$ (Student's t-test). Hcy: homocysteine; NF-κB: nuclear factor-kappaB.

Figure 6. Effect of chronic administration of homocysteine on acetyl cholinesterase activity in the hippocampus of rats. Results are expressed as mean ± SD for six animals per group. Different from control, $^{*}p<0.05$ (Student's t-test). Hcy: homocysteine.

Figure 1

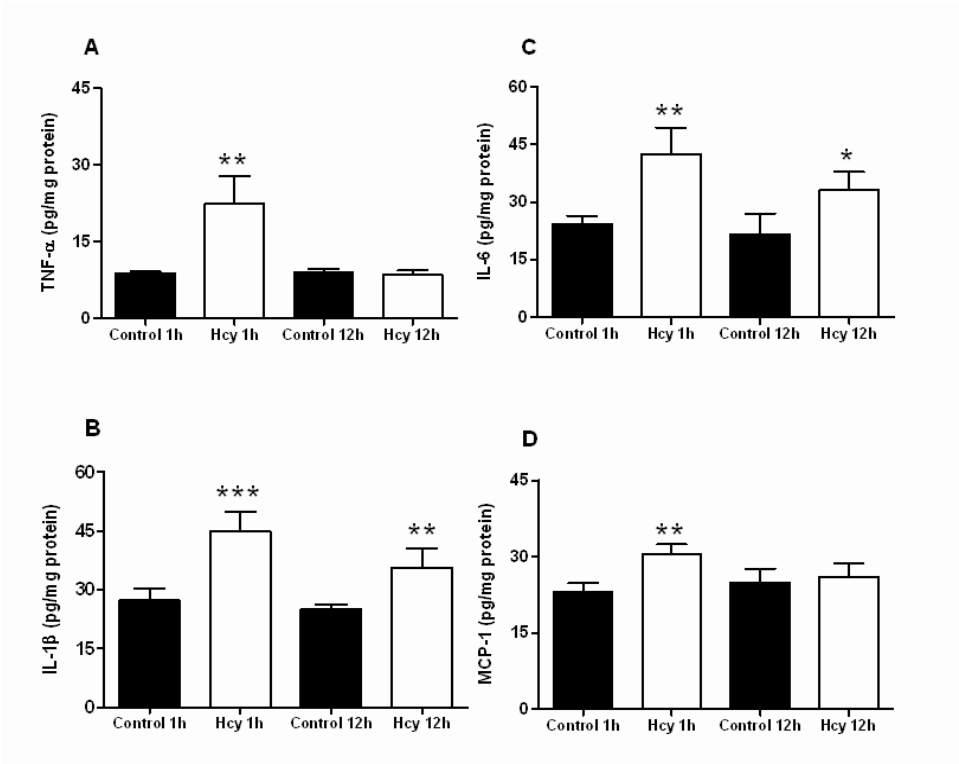


Figure 2

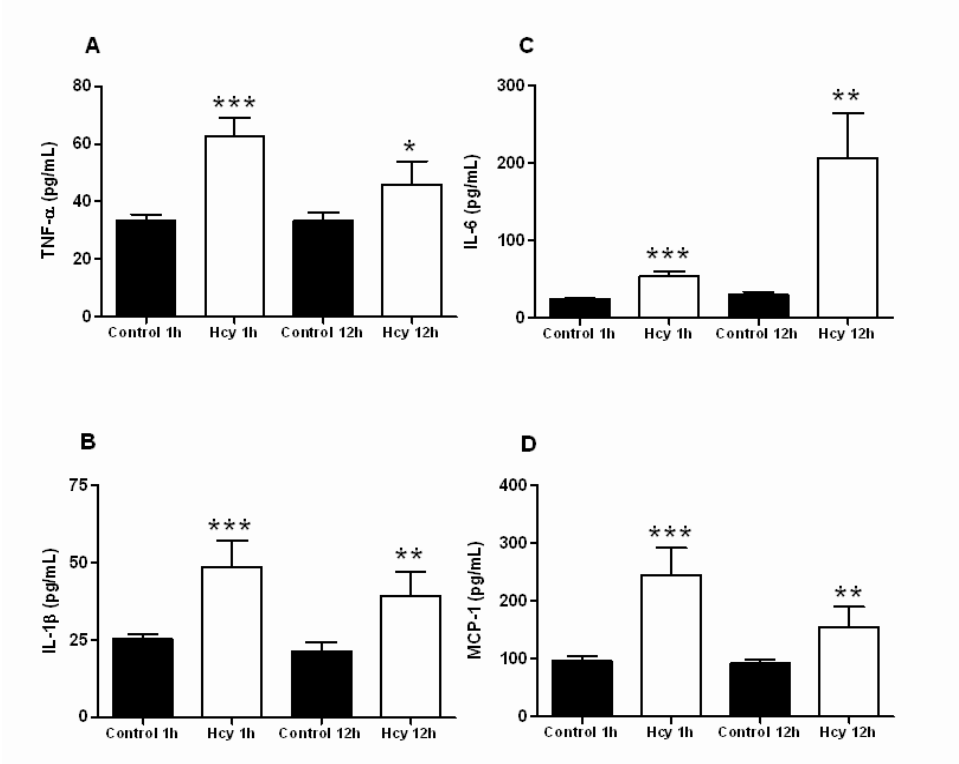


Figure 3

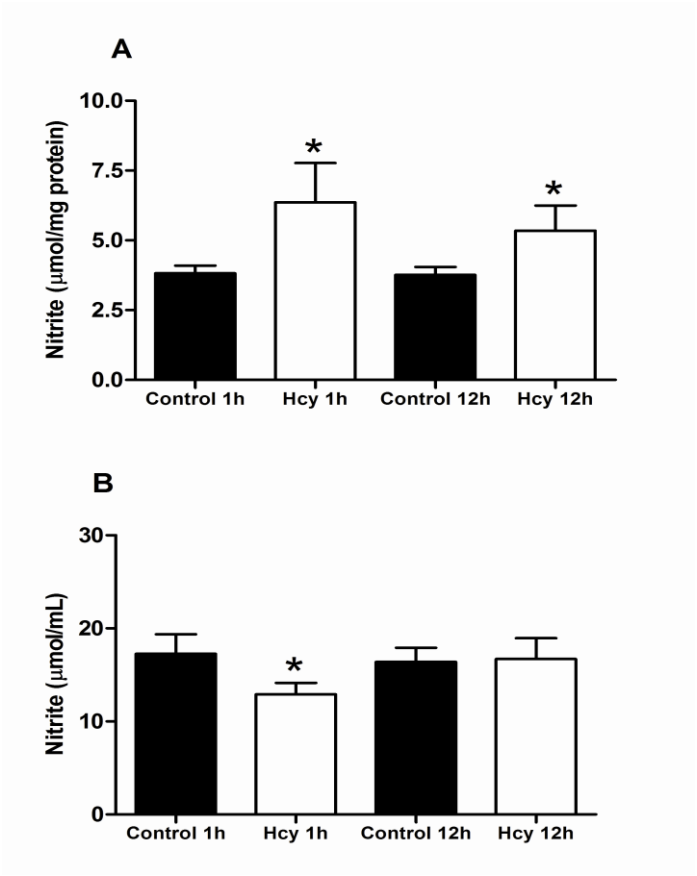


Figure 4

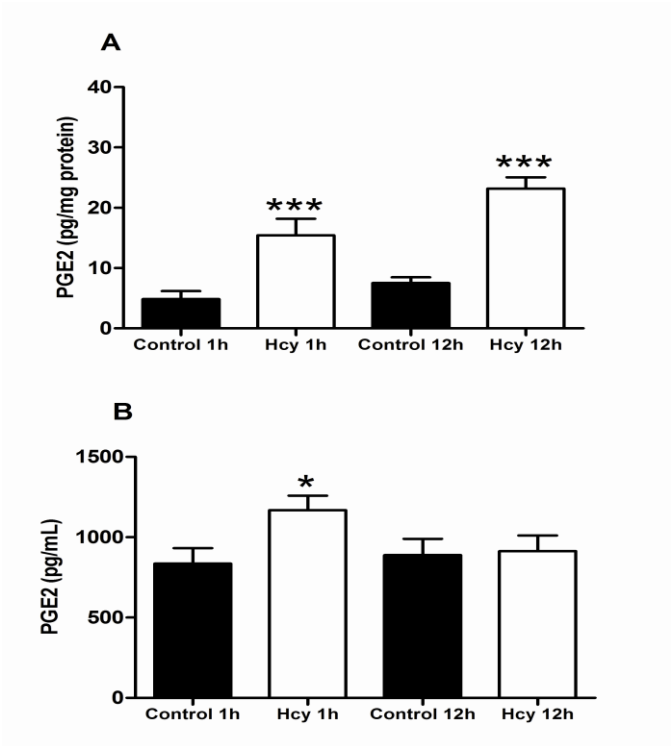


Figure 5

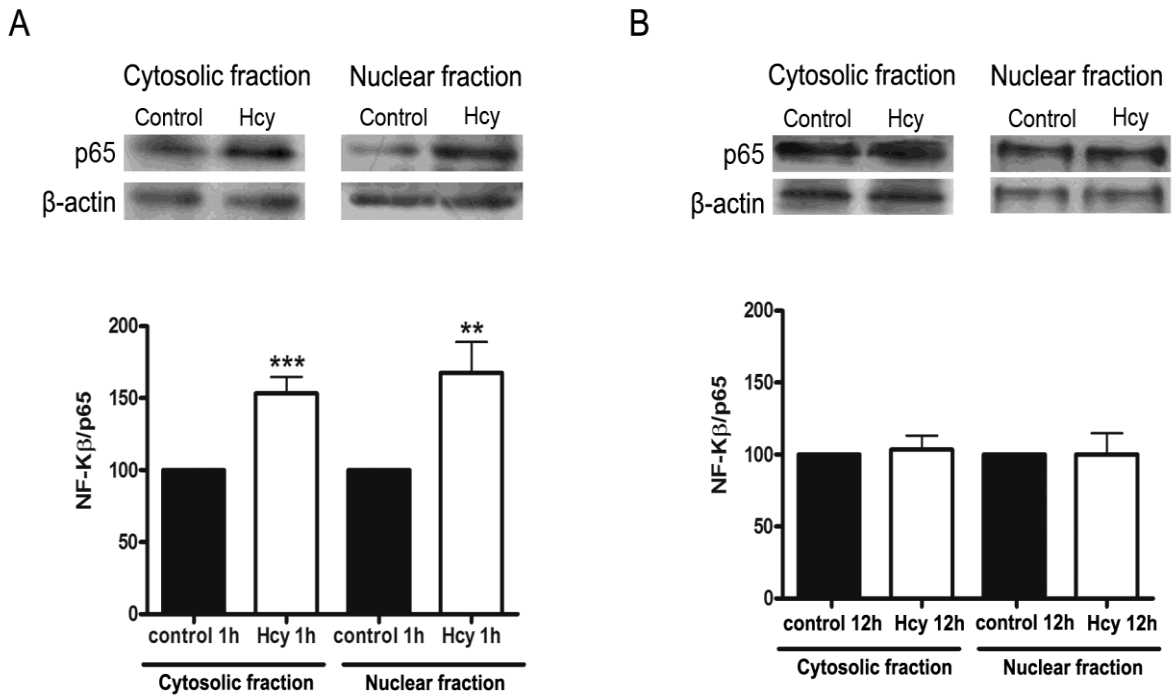
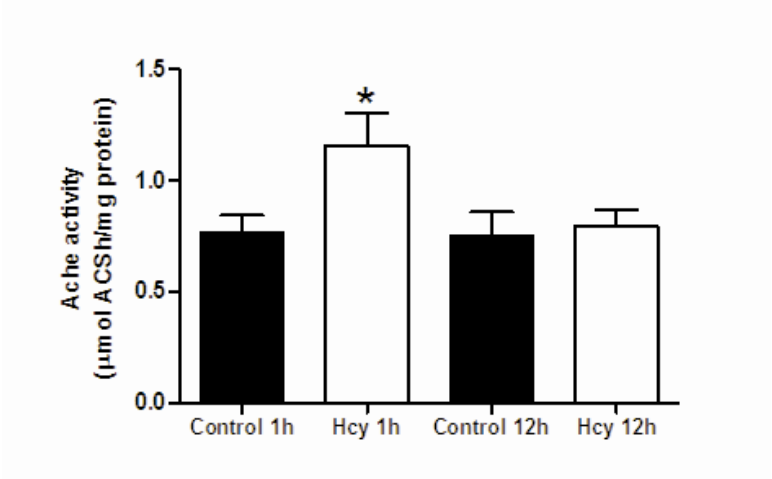


Figure 6



Capítulo III

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Acute hyperhomocysteinemia alters the coagulation system and oxidative status in the blood of rats

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Abstract In the present study, we investigated the effect of the acute administration of homocysteine (Hcy) on parameters of the coagulation system, as well as fibrinogen and nitrite levels in the blood of rats. In addition, we evaluated the effect of acute hyperhomocysteinemia on thiobarbituric acid-reactive substances in plasma and on antioxidant enzymes activities (superoxide dismutase, catalase, and gluthatione peroxidase) in the erythrocytes of rats. Wistar rats, aged 29 days, received a single subcutaneous dorsal injection of saline (control) or Hcy (0.6 μmol/g body weight). Fifteen minutes, 1 h, 6 h or 12 h after the injection, the rats were euthanized and the blood, plasma, and erythrocytes were collected. Results showed that Hcy significantly increased platelet count in the blood and plasma fibrinogen levels of rats at 15 min and 1 h, but not at 6 h and 12 h, when compared with the control group. Prothrombin time, activated partial thromboplastin time, and nitrite levels significantly decreased in plasma at 15 min and 1 h, but not at 6 h and 12 h after Hcy administration. In addition, hyperhomocysteinemia increased thiobarbituric acid-reactive, an index of lipid peroxidation, in plasma at 15 min and 1 h; decreased the superoxide dismutase and gluthatione peroxidase activity, and increased the catalase activity at 15 min in erythrocytes of rats, suggesting that acute Hcy administration may alter the oxidative status in the blood of rats. Our findings suggest that hypercoagulability and oxidative stress can occur after acute hyperhomocysteinemia, possibly in association, at least in part, with the vascular dysfunction and thromboembolic complications observed in homocystinuric patients.

Keywords Homocysteine · Coagulation system · Oxidative stress · Nitric oxide

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Introduction

Classical homocystinuria is an inborn error of metabolism caused by a severe deficiency of cystathionine β-synthase (CBS, EC 4.2.1.22) activity, resulting in the tissue accumulation of homocysteine (Hcy) [1]. It has been reported that Hcy levels are increased in patients with essential hypertension, stroke or atherosclerosis [2, 3]. Plausible mechanisms by which Hcy might contribute to atherogenesis include the induction of platelet activation and enhanced coagulability, increased smooth muscle cell proliferation, cytotoxicity, increased oxidative stress, induction of endothelial dysfunction, and stimulation of low-density lipoprotein oxidation [4, 5]. However, the molecular mechanisms by which increased circulating Hcy causes vascular injury and promotes thrombosis remain elusive [6].

The blood coagulation system constitutes numerous complex reactions of platelets, coagulation factors, and

fibrinolysis factors [7, 8]. The cascade of reactions leading to blood coagulation consists of two main pathways. One is the "extrinsic pathway," initiated by the reaction of tissue thromboplastin, and followed by factors III (tissue thromboplastin), VII, and X. The other is the "intrinsic pathway," which is initiated by factor XII activation followed by factors XI, IX, and VIII. The extrinsic (activated factor VII) or intrinsic (factor Xa) pathways activate factor X, in the presence of phospholipid, Ca^{2+}, and factor V. Factor Xa catalyzes the reaction from prothrombin to thrombin, which is involved in the transformation of fibrinogen into fibrin [7]. Since these factors are synthesized in the liver, any agent that impairs liver function may impair the production of coagulation factors [7].

Hemostatic abnormalities can lead to excessive bleeding, thrombosis or other cardiovascular diseases. Recently, it has been shown that Hcy induces changes in hemostasis, including blood clotting and fibrinolysis [9]. Evidence also suggests that Hcy impairs nitric oxide (NO) release from endothelial cells [10] and platelets [11], enhances arachidonic acid release and reactive oxygen species (ROS) formation in platelets [12], as well as stimulating smooth muscle cell proliferation [13]. We previously showed that Hcy induces oxidative stress in the brain of rats, reducing antioxidant defenses, and increasing lipid peroxidation [14–16]. In this context, Kolling et al. [17] showed that chronic hyperhomocysteinemia increased lipid peroxidation, ROS, and decreased enzymatic antioxidant defenses and nitrite levels in the heart of rats.

Considering that hyperhomocysteinemia has been associated with an increased risk of arterial thrombosis, as well as venous thromboembolism, the main objective of the present study was to investigate the effect of acute Hcy administration on some parameters of the blood coagulation system, such as platelet count, the blood clotting time [prothrombin time (PT) and activated partial thromboplastin time (APTT)], as well as fibrinogen and nitrite levels in blood and/or plasma of rats. In addition, we investigated the effect of acute hyperhomocysteinemia on some parameters of oxidative stress, namely thiobarbituric acid-reactive (TBARS) and activities of antioxidant defenses, superoxide dismutase (SOD), catalase (CAT), and gluthathione peroxidase (GPx) in plasma and/or erythrocytes of rats.

Materials and methods

Animals and reagents

Seventy-four Wistar rats, aged 29 days, were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals 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.

Acute Hcy treatment

Rats received a single subcutaneous dorsal injection of saline solution (control) or *D,L*-Hcy (0.6 μmol/g body weight) dissolved in saline and buffered to pH 7.4 according to Streck et al. [18]. Rats subjected to this treatment achieved plasma Hcy levels similar to those found in classical homocystinuric patients (500 μM) [1]. Hcy crosses the blood brain barrier and presents a peak in the plasma 15 min after subcutaneous injection [18].

Blood collection for coagulation tests

Fifteen minutes, 1, 6, and 12 h after acute Hcy administration, the animals were anesthetized with ethyl ether and blood was collected from the heart through direct punction, in syringes with sodium citrate (1:9) [19]. Blood collected with sodium citrate was centrifuged at 2000×*g* for 10 min, at 24°C and the plasma obtained was used for coagulation tests (PT, APTT), fibrinogen, and nitrite levels.

Erythrocyte separation for oxidative stress parameters

Whole blood was collected and transferred to heparinized tubes for erythrocyte separation. Blood samples were centrifuged at 1000×*g* and plasma was removed for TBARS determination. Erythrocytes were washed three times with cold saline solution (0.153 mol/l sodium chloride). Lysates were prepared by the addition of 1 ml of distilled water to 100 μl of washed erythrocytes and frozen at −80°C until determination of the antioxidant enzyme activities. For antioxidant enzyme activity (SOD, CAT, and GPx), erythrocytes were frozen and thawed three times to break the erythrocyte membrane and release enzymes, and centrifuged at 13.500×*g* for 10 min. The supernatant was diluted in order to contain ∼0.5 mg/ml of protein.

Platelet count

Blood collected with citrate was analyzed in a blood counter (Coulter® T890, USA) to obtain the platelet count.

Prothrombin time (PT)

The PT test was performed using the commercial kit, Soluplastin®, provided by Wiener Lab (Rosario, Argentina). In brief, 100 µl of plasma kept in a water bath at 37°C were added to 200 µl of a solution containing rabbit brain thromboplastin, 0.0125 mol/l calcium chloride, and 0.1 mol/l calcium sodium. The time elapsed between the addition of plasma and clot formation was analyzed in an eletromechanical ST4 coagulation analyzer (Diagnostica Stago, Parsippany, NJ) according to the manufacturer's standard protocol.

Activated partial thromboplastin time (APTT)

The APTT test was performed using the commercial APTTest® kit, provided by Wiener Lab (Rosario, Argentina). For this test, 100 µl of plasma and 100 µl of cefalin were maintained in a water bath at 37°C for 3 min, in the same tube. Subsequently, 100 µl of 0.025 mol/l calcium chloride were added to the solution and the time until clot formation was measured in an eletromechanical ST4 coagulation analyzer (Diagnostica Stago, Parsippany, NJ) according to the manufacturer's standard protocol.

Fibrinogen test

The fibrinogen test was performed using the commercial kit Fibrinógeno®, provided by Wiener Lab (Rosario, Argentina). For this test, 40 µl of plasma is mixed with 360 µl of 0.05 M imidazole buffer and maintained in a water bath at 37°C. After 2 min, 200 µl of plasma solution were added to 100 µl of reconstituted thrombin (100 UNIH/ml) and the time until clot formation was analyzed in an eletromechanical ST4 coagulation analyzer (Diagnostica Stago, Parsippany, NJ) according to the manufacturer's standard protocol.

Nitrite assay

Nitrite is the stable end product of the auto-oxidation of NO in aqueous solution [20]. Nitrite levels were measured using the Griess reaction; 100 µl of plasma were mixed with 100 µl Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature. The absorbance was measured on a microplate reader at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards [21].

Thiobarbituric acid-reactive substances (TBARS)

TBARS were determined according to the method described by Ohkawa et al. (1979). For determination, plasma in 1.15% KCl was mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid, and heated in a boiling water bath for 60 min. TBARS were determined by the absorbance at 535 nm in a Beckman DU®640 Spectrophotometer. The results were reported as ηmol TBARS/mg protein [22].

Superoxide dismutase assay (SOD)

The SOD activity assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on $O_2^{\bullet -}$, a substrate for SOD. The inhibition of auto-oxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol auto-oxidation and the specific activity is reported as SOD units/mg protein [23].

Catalase assay (CAT)

CAT activity was assayed using a double-beam spectrophotometer with temperature control (Hitachi U-2001). This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/ml. One CAT unit is defined as 1 mmol of H_2O_2 consumed per minute and the specific activity is reported as CAT units/mg protein [24].

Glutathione peroxidase assay (GPx)

GPx activity was measured by the method of Wendel (1981), except for the concentration of NADPH, which was adjusted to 0.1 mM after previous tests performed in our laboratory. Tert-butylhydroperoxide was used as substrate. NADPH disappearance was continuously monitored with a spectrophotometer at 340 nm for 4 min. One GPx unit is defined as 1 mmol of NADPH consumed per minute and specific activity is reported as GPx units/mg protein [25].

Protein determination

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard [26].

Statistical determination

All the data are expressed as the mean ± standard deviation (SD). Data were analyzed by Student's t test for unpaired samples. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $P < 0.05$.

Results

We first investigated the effect of acute Hcy administration on platelet count in rats. Figure 1 shows that platelet count was significantly increased in the blood of rats (15 min: [$t(10) = 6.26$; $P < 0.001$]; 1 h: [$t(10) = 3.84$; $P < 0.01$]), but not at 6 h [$t(10) = 2.10$; $P > 0.05$] and 12 h [$t(10) = 1.24$; $P > 0.05$] after the last injection of this amino acid, as compared with the control group.

Next, we evaluated the effects of Hcy administration on PT, APTT, and fibrinogen levels in the plasma of rats. Figure 2a shows that a single Hcy injection significantly decreased PT (15 min: [$t(10) = 9.10$; $P < 0.001$]; 1 h: [$t(10) = 7.06$; $P < 0.001$]). As can be observed in Fig. 2b, Hcy significantly decreased APTT (15 min: [$t(10) = 6.01$; $P < 0.001$]; 1 h: [$t(10) = 4.78$; $P < 0.001$]), when compared with the control group. Conversely, administration of Hcy did not alter PT and APTT at 6 h: ([$t(10) = 2.15$; $P > 0.05$]; [$t(10) = 1.83$; $P > 0.05$]) and 12 h: ([$t(10) = 1.34$; $P > 0.05$]; [$t(10) = 0.53$; $P > 0.05$]), respectively. Figure 3 shows that Hcy administration significantly increased fibrinogen levels (15 min: [$t(10) = 4.94$; $P < 0.01$]; 1 h: [$t(10) = 13.01$; $P < 0.001$]), as compared with the control group. In contrast, Hcy did not change fibrinogen levels at 6 h [$t(10) = 0.21$; $P > 0.05$] and 12 h [$t(10) = 1.54$; $P > 0.05$] after acute hyperhomocysteinemia.

We measured the effect of acute Hcy administration on nitrite levels in plasma of rats. Figure 4 shows that nitrite levels significantly decreased in plasma at 15 min [$t(10) = 2.75$; $P < 0.05$] and 1 h [$t(10) = 6.29$; $P < 0.001$] after Hcy administration when compared with the control group, but not at 6 h [$t(10) = 1.02$; $P > 0.05$] and 12 h [$t(10) = 1.20$; $P > 0.05$] after acute Hcy injection.

We also evaluated the effect of acute Hcy administration on TBARS, an index of lipid peroxidation, in the plasma of rats. Figure 5 shows that TBARS significantly increased in plasma at 15 min [$t(10) = 6.73$; $P < 0.001$] and 1 h [$t(10) = 2.87$; $P < 0.05$] after acute hyperhomocysteinemia, when compared with the control group.

Finally, we verified the effect of acute Hcy injection on enzymatic antioxidant defenses (SOD, CAT, and GPx). Figure 6a shows a significant decrease in the SOD activity in erythrocytes of Hcy-treated rats at 15 min [$t(10) = 6.62$; $P < 0.001$], but not at 1 h [$t(10) = 1.31$; $P > 0.05$] after Hcy injection. In contrast, Hcy significantly increases CAT activity at 15 min [$t(10) = 4.22$; $P < 0.001$], but not at 1 h [$t(10) = 0.44$; $P > 0.05$], after acute injection of this amino acid. To verify whether another antioxidant enzyme that reduces H_2O_2 was compensating for the observed imbalance between SOD and CAT, we determined GPx activity. As shown in Fig. 6c, acute Hcy administration decreased GPx activity at 15 min [$t(10) = 4.84$; $P < 0.001$], but not at 1 h [$t(10) = 0.82$; $P > 0.05$] after Hcy administration in the erythrocytes of rats.

Discussion

Classical homocystinuria is a congenital thrombotic disorder in which affected patients present elevated plasma levels of Hcy [1]. It has been shown that increased levels of this amino acid promote endothelial cell damage, platelet hyperactivity, and the production of abnormal clotting factors, leading to the development of thromboembolic plaques in the coronary, carotid, and peripheral vascular systems [4, 5, 27]. However, the mechanisms by which thrombotic events occur in these patients are unknown.

Previous studies in vitro have identified a number of possible mechanisms that may contribute to Hcy induced disease, including endothelial cell injury [28], increased adhesion molecule expression [29], increased monocyte

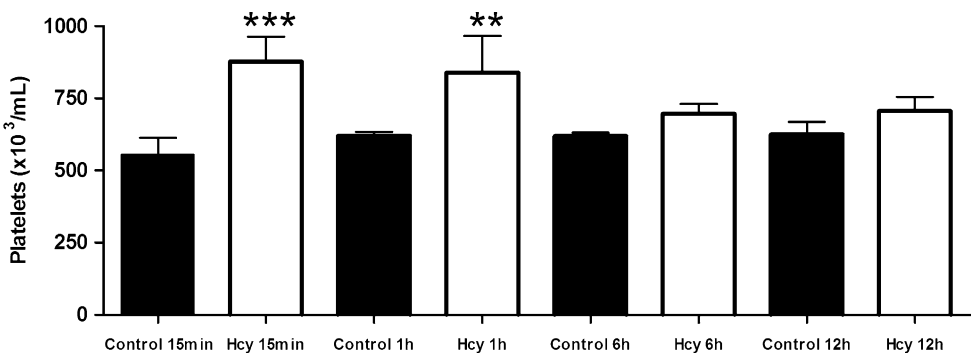

Fig. 1 Effect of acute administration of Hcy on platelet count in the blood of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's t test). *Hcy* homocysteine

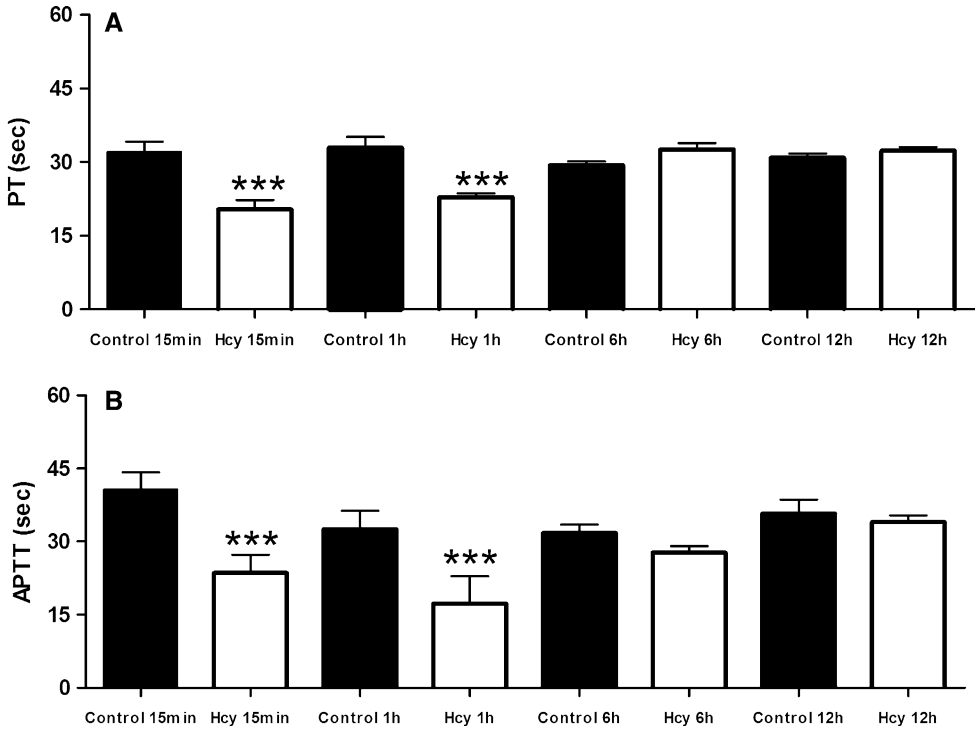

Fig. 2 Effect of acute administration of Hcy on PT (**a**) and APTT (**b**) in the plasma of rats. Results are expressed as means ± SD for six animals per group. Different from control, ***$P < 0.001$ (Student's t test). *Hcy* homocysteine

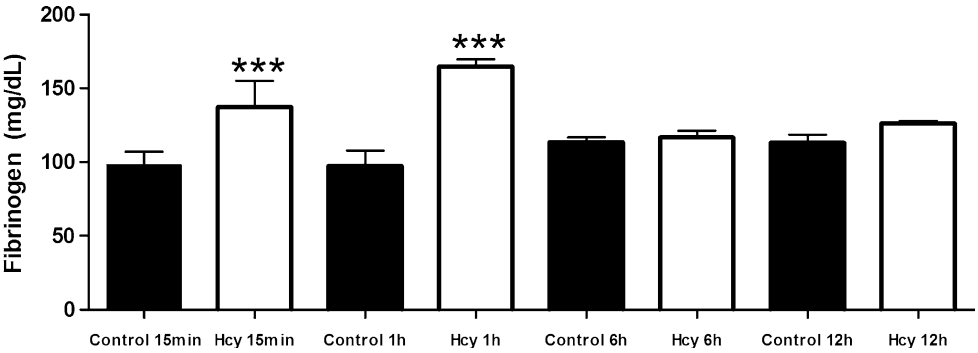

Fig. 3 Effect of acute administration of Hcy on fibrinogen levels in plasma of rats. Results are expressed as mean ± SD for six animals per group. Different from control, ***$P < 0.001$ (Student's t test). *Hcy* homocysteine

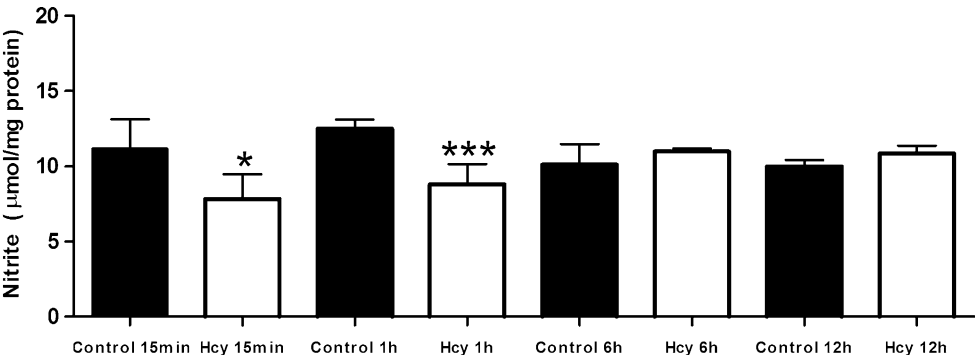

Fig. 4 Effect of acute administration of Hcy on nitrite levels in plasma of rats. Results are expressed as means ± SD for six animals per group. Different from control, *$P < 0.05$; ***$P < 0.001$ (Student's t test). *Hcy* homocysteine

and T-cell binding to endothelial cells [30], reduced prostacyclin production [31] and NO synthesis [32], reduced endothelium-dependent relaxation [33], induction of thrombosis through enhanced factor V activation [34], as well as increased oxidative stress [14, 35, 36]. Taken together, these findings demonstrate the multi-factorial nature of Hcy induced vascular disease; however, many questions remain to be answered.

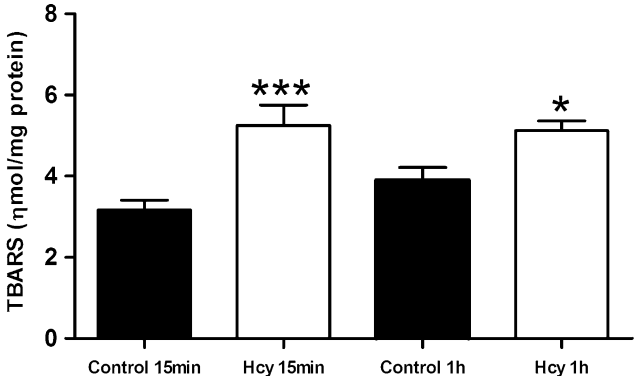

Fig. 5 Effect of acute administration of Hcy on thiobarbituric acid reactive substance levels in the plasma of rats. Results are expressed as means ± SD for six animals per group. Different from control, $*P < 0.05$; $***P < 0.001$ (Student's *t* test). *Hcy* homocysteine

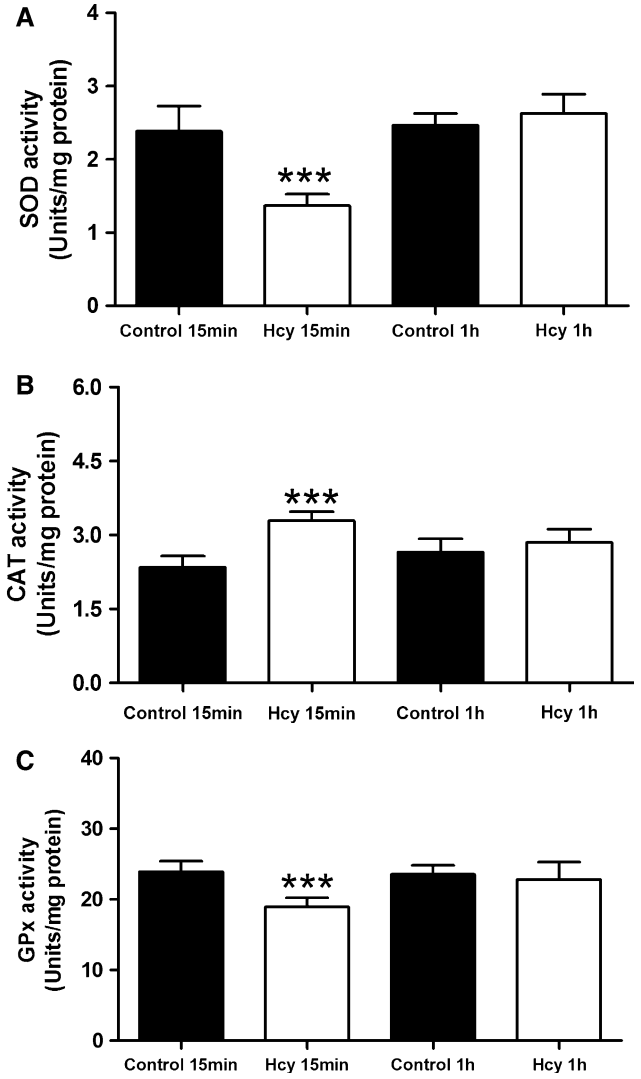

Fig. 6 Effect of chronic Hcy administration on superoxide dismutase (**a**), catalase (**b**) and glutathione peroxidase activity (**c**) in erythocytes of rats. Results are expressed as means ± SD for six animals per group. Different from control, $***P < 0.001$ (Student's *t* test). *Hcy* homocysteine

In the present study, we initially investigated the effects of acute hyperhomocysteinemia on the coagulation system by determining the platelet count in blood of rats. Results showed that Hcy increased platelet count at 15 min and 1 h after acute Hcy administration. However, from 6 and 12 h after acute hyperhomocysteinemia onwards, there was no effect on this parameter. It is possible that the increase in the platelet count probably depended on Hcy, since a previous study showed that this amino acid presents a peak in the plasma at 15 min after injection, returning to baseline levels after 12 h [18].

Although several studies have shown that Hcy induces changes in the coagulation response [35, 37], relatively few studies have directly addressed the influence of Hcy on platelet function. Platelets exert a crucial function in hemostasis, wound repair, and the formation of vascular plugs, underlying thrombotic disease, such as stroke and myocardial infarction [8]. Mohan et al. (2008) suggest that platelet activation and hypercoagulability occur after exposure to Hcy, especially in patients with critical limb ischemia [38]. In addition, abnormalities in platelet adhesion and aggregation have been described in some homocystinuric patients [1]. In this context, it has been shown that patients with severe hyperhomocysteinemia present increased circulating plasma thromboxane metabolites, indicating platelet activation [39]. Furthermore, recent studies have demonstrated that Hcy treatment increased platelet aggregation induced by ADP [40].

In order to investigate a possible alteration in the coagulation system caused by acute Hcy administration, we also evaluated the blood clotting time, PT, and APTT. Results showed that Hcy promotes a decrease in the blood clotting time (PT and APTT) in the plasma of rats sacrificed at 15 min and 1 h, but not at 6 and 12 h, after acute Hcy injection, indicating that this amino acid provokes a hypercoagulable state in the plasma of rats. In agreement with these data, it has been shown that hyperhomocysteinemic rats present an increase in factor VIII in the plasma [41]. This factor participates in the activation of factor V and its increased levels are associated with the risk factor for venous thromboembolism, independently of the acute phase reaction [42–44]. In this context, Lijfering et al. (2007) showed that the increased risk of cardiovascular diseases in hyperhomocysteinemia is mainly related to elevated coagulation factor VIII levels [45]. Other mechanisms that may contribute to the hypercoagulable status in homocystinuria include activation of factors V and XII [34]. On the other hand, Maclean et al. (2010) did not observe significant alteration of coagulation parameters in transgenic mouse model of CBS-deficient homocystinuria [46].

Abnormalities of the extrinsic and intrinsic coagulation pathways can be screened by routine PT and APTT parameters [7]. PT and APTT are used as screening tests for factors VII, X, V, and II of the extrinsic [47] and intrinsic pathways for factors XII, XI, IX, VII, X, V, and II, respectively [48]. It has been demonstrated that hyperhomocysteinemia changes whole blood coagulation by increasing the velocity and the firmness of the clot formed, and by prolonging the initiation phase of the coagulation in rats [49]. On the other hand, a prolongation of APTT has been described previously in hyperhomocysteinemia rabbits, whereas the PT was unaffected [50]. The prolongation of the initiation phase of coagulation may seem paradoxical because hyperhomocysteinemia is associated with thrombotic disease [51].

In this study, we demonstrated an increase in the fibrinogen levels in the plasma of rats at 15 min and 1 h after acute Hcy administration. Fibrinogen is a glycoprotein that is synthesized in the liver, and found in plasma and the α-granules of platelets. Fibrinogen plays a central role in hemostasis by forming a cross-linking meshwork of fibrin [52]. Elevations in fibrinogen levels have been associated with an increased risk of thrombotic disease [53]. In agreement with our results, it has been reported that rabbits chronically injected with Hcy present an acquired dysfibrinogenemia, resulting in prolonged clotting times, increased resistance to plasmin lysis and structural alterations in fibrin network [50]. Increased oxidant stress also favors a procoagulant state by influencing the function of key mediators of fibrinolysis [54]. Fibrinogen is a coagulation factor related with hypercoagulation [54] and an acute phase protein for inflammatory reaction [55]. Therefore, the increase in fibrinogen levels may be associated to both oxidative stress and inflammation induced by Hcy, since previous study in our group also demonstrated that acute administration of Hcy increases inflammatory parameters, such as TNF-α and IL-6, at 15 min after the last injection of this amino acid [56]. In the present study, we demonstrated that all parameters of coagulation system evaluated changed rapidly after acute Hcy injection, suggesting that the effects of Hcy may be closely related to the high plasma levels of this amino acid, which achieve the peak as soon at 15 min after injection [18].

Considering that NO plays an important role in a number of physiological processes and has been implicated in a number of vascular diseases [57], we also measured the nitrite levels in the plasma of rats subjected to acute Hcy administration. We observed a decrease in the levels of nitrite in the plasma of rats sacrificed at 15 min and 1 h, but not at 6 and 12 h after Hcy injection. These results are in agreement with those of Zhang et al. (2002), who showed that high Hcy levels in serum suppress the NO released from the endothelium [58]. NO is produced from L-arginine in many cell types through the action of nitric oxide synthase (NOS). Furthermore, NO release by the endothelium regulates blood flow, inflammation, and platelet aggregation, and consequently its disruption during endothelial dysfunction can decrease plaque stability and encourage the formation of atherosclerosis lesions and thrombi [59]. There is some controversy as to the effects of hyperhomocysteinemia on NO production. In this context, it has been shown that hyperhomocysteinemia both up-regulates [60] and down-regulates NO production [61]. On the other hand, Leoncini et al. (2003) demonstrated that high plasma Hcy levels increased Ca^{2+} levels and reduced NO formation, a potent antiaggregating agent of platelets [62]. In this context, da Cunha et al. (2010) showed that acute hyperhomocysteinemia increased nitrite levels in the cerebral cortex and hippocampus of rats [56].

It has been reported that the oxidative effects of Hcy can cause perturbations in the vasculature [10, 32]. The main molecules responsible for decrease of NO level in hyperhomocysteinemic patients are probably ROS [57]. Fischer et al. (2003) showed that peroxinitrite, which is produced in the reaction of NO with $O_2^{\bullet -}$, increases the protein nitration [63]. Nitrosation of Hcy may be responsible for a decrease in the levels of NO, because Hcy reacts with NO to form S-nitrosohomocysteine [64].

Since Hcy may exert cellular damage through oxidative mechanisms [14, 35, 36], we also evaluated the effect of hyperhomocysteinemia on TBARS, a parameter considered to be an index of lipid peroxidation in the plasma of rats. We observed that acute hyperhomocysteinemia increased TBARS levels at 15 min and 1 h after the last injection of Hcy. Hcy is readily oxidized in plasma, mainly as a consequence of auto-oxidation leading to the formation of Hcy, Hcy-mixed disulfides, and Hcy thiolactone. During the oxidation of its sulfhydryl group, ROS may be generated, such as $O_2^{\bullet -}$, H_2O_2, or $OH^{\bullet}$, and it has been suggested that these species may have harmful effects [65–67]. Moreover, this ROS generated during Hcy oxidation may initiate lipid peroxidation [68]. Furthermore, it has been shown that Hcy induces a consistent oxidative stress in the hippocampus, parietal cortex and liver of rats, increasing lipid peroxidation, and reducing enzymatic and non-enzymatic antioxidant defenses [14, 16, 69, 70].

Finally, we evaluated the effect of acute hyperhomocysteinemia on the activities of antioxidant defenses (SOD, CAT, and GPx) in erythrocytes, which compose an efficient system responsible for removing ROS [71]. Our results show that animals exposed to acute hyperhomocysteinemia present an imbalance between SOD and CAT activities, expressed as a decrease in SOD activity and an increase in CAT activity in erythrocytes of rats. The increase in antioxidants enzymes is very important to protect the organism against increased ROS production;

however, a balance in these enzymes is also needed [71]. It is well known that SOD and CAT have sequential functions in ROS removal, by catalyzing $O_2^{\bullet -}$ dismutation, followed by H_2O_2 conversion to H_2O and O_2, respectively [71]. Taken together, the decrease in GPx activity at 15 min, compromising the efficiency of reactive species detoxification, could lead to oxidative damage to biomolecules. In addition, it has been demonstrated that acute Hcy administration decreases the radical-trapping antioxidant potential (TRAP) in the hippocampus of rats, and that vitamins E and C completely prevented these activities, indicating that the participation of oxidative stress is probably involved in the effects of Hcy [36].

In conclusion, our findings suggest that acute hyperhomocysteinemia causes hypercoagulability in the plasma of rats. These results could be responsible for the modification of hemostasis observed in homocystinuric patients. In addition, our study shows that Hcy increased fibrinogen levels, a molecule that plays a central role in hemostasis and the inflammatory reaction. We also demonstrated that Hcy decreases nitrite levels, possibly contributing to endothelial dysfunction and vascular response impairment. Furthermore, Hcy promotes lipid peroxidation and alters the antioxidant defenses, which may be implicated in the development of atherosclerosis and thrombosis. The mechanism of Hcy action on the homeostasis process is complex and still unclear; however, we suggest that hypercoagulability could be a consequence of many confluent mechanisms, such as oxidative stress, which might contribute to the elevated atherothrombotic risk in patients with increased plasma Hcy concentrations.

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EVIDENCE THAT AKT AND GSK-3β PATHWAY ARE INVOLVED IN ACUTE HYPERHOMOCYSTEINEMIA

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Abstract

Homocysteine is a neurotoxic amino acid that accumulates in several disorders including homocystinuria, neurodegenerative and neuroinflammatory diseases. In the present study we evaluated the effect of acute and/or chronic hyperhomocysteinemia on Akt, NF-κβ/p65, GSK-3β, as well as *Tau* protein in hippocampus of rats. For acute treatment, rats received a single injection of homocysteine (0.6 µmol/g body weight) or saline (control). For chronic treatment, rats received daily subcutaneous injections of homocysteine (0.3-0.6 µmol/g body weight) or saline (control) from the 6^{th} to the 28^{th} days-of-age. One or 12 h after the last injection, rats were euthanized, the hippocampus was removed and samples were submitted to electrophoresis followed by Western blotting. Results showed that acute hyperhomocysteinemia increases Akt phosphorylation, cytosolic and nuclear immunocontent of NF-κβ/p65 subunit and *Tau* protein phosphorylation, but reduces GSK-3β phosphorylation at 1 h after homocysteine injection. However, 12 h after acute hyperhomocysteinemia there is no effect on Akt and GSK-3β phosphorylation. Furthermore, chronic hyperhomocysteinemia did not alter Akt and GSK-3β phosphorylation at 1 h and 12 h after the last administration of this amino acid. Our data showed that Akt, NF-κβ/p65, GSK-3β and *Tau* protein are activated in hippocampus of rats subjected to acute hyperhomocysteinemia, suggesting that these signaling pathways may be, at least in part, important contributors to the neuroinflammation and/or brain dysfunction observed in some hyperhomocystinuric patients.

Keywords: Hyperhomocysteinemia; Akt; NF-κβ/p65 subunit; GSK-3β; Tau protein.

1. Introduction

Homocysteine (Hcy), a methionine-derived sulphur amino acid, has been associated with several disorders that affect the central nervous system (CNS), such as epilepsy (Sachdev 2004; Herrmann et al. 2007), stroke (Obeid et al. 2007), neuropsychiatric and neurodegenerative diseases (Diaz-Arrastia 2000; Bottiglieri 2005), as well as homocystinuria, an inborn error of metabolism biochemically characterized by cystathionine β-synthase (CBS) (E.C. 4.2.1.22) deficiency (Mudd et al. 2001).

The underlying mechanism by which Hcy exert its neurotoxic effects remains unexplained, however, some hypotheses have been described. It has been shown that Hcy induces oxidative stress by activation of glutamatergic receptors, with consequent reactive oxygen species (ROS) generation (Kim and Pae 1996; Zhang et al. 1998; Jara-Prado et al. 2003; Zieminska and Lazarewicz 2006), or by auto-oxidation to Hcy and other disulphides releasing superoxide anion ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) (Kim and Pae 1996; Ho et al. 2002; Faraci and Lentz 2004). In addition, we previously showed that Hcy increased cytokines in brain of rats, suggesting that inflammation might be associated with the neuronal dysfunction observed in hyperhomocystinuric patients (da Cunha et al. 2010).

Cytokines play an important role in the abnormal immune response, and recent studies have confirmed that regulation and release of cytokines are closely associated with the phosphoinositide 3-kinase (PI3K) signaling pathway (Goyal et al. 2002; Selvaraj et al. 2003). Activated Akt, also known as protein

kinase B, is released from the cell membrane into the cytoplasm or nucleus to further transmit the signals and execute its biological functions (Lin et al. 2001). Thus, Akt phosphorylation can be used as an indicator of PI3K activity (Lin et al. 2001; Edstrom and Ekstrom 2003). One way by which active Akt mediates its effects is by phosphorylating proteins, such as, nuclear factor-kappaB (NF-κB), glycogen synthase kinase-3 (GSK3), CREB, Bad and caspase 9 (Coffer et al. 1998; Downward 1998; Datta et al. 1999).

NF-κB is formed by homo or heterodimers comprising members of the Rel family of proteins (p50/p105, p52/p100, p65/RELA, c-Rel and RelB) which form, upon non-stimulated conditions, a ternary and inactive cytosolic complex by interacting with inhibitory proteins of the IkB family. Upon stimulation by cytokines (TNF-α and IL-1β), growth factors and other stressor stimuli, IkBs are phosphorylated by IKK (IkB kinase) proteins thus releasing the active NF-kB, which translocates into nucleus and binds to DNA sequences in gene promoters. NF-kB binding to DNA modulates the expression of a wide range of genes, including antimicrobial peptides, cytokines, chemokines, stress-response proteins, anti-apoptotic proteins and adhesion molecules (Li and Verma, 2002).

GSK3 designates two isoforms, GSK-3α and GSK-3β that are ubiquitously expressed, highly homologous and usually have equivalent actions (Jope and Johnson 2004). GSK-3β contributes to the neuropathology of Alzheimer`s disease by regulating the phosphorylation of the microtubule-associated protein *Tau* that is the predominant component of neurofibrillary tangles in Alzheimer`s disease (Jope and Johnson 2004). More recently it has become evident that GSK-3β is a crucial, and often central, component of many

cellular functions, contributing to the regulation of apoptosis, cell cycle, cell polarity and migration, gene expression, and many other functions (Grimes and Jope 2001; Jope and Johnson 2004). This multi-tasking is achieved by the many substrates phosphorylated by GSK-3β and the convergence on GSK-3β of many regulatory intracellular signaling pathways (Jope and Johnson 2004). A new cellular function regulated by GSK-3β was identified by recent findings showing that GSK-3β is a vital factor in the inflammation process (Martin et al. 2005).

Since Hcy and inflammatory parameters seem to be associated with the pathogenesis of several diseases (Welch and Loscalzo 1998; Weiss et al. 2003; Gori et al. 2005), in the present study we investigated the effect of acute and chronic hyperhomocysteinemia on the signaling mechanism through analysis of Akt, NF-kB/p65, GSK-3β and *Tau* protein in rat hippocampus.

2. Materials and Methods

2.1 Animals and reagents

Seventy-eight 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.

Acrylamide, bisacrylamide, SDS, and β-mercaptoethanol used in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit IgG peroxidase-conjugated and reagents to detect chemioluminescence (ECL) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Hybond-C nitrocellulose membranes were from Hybond-ECL-(Hybond- ECL- nitrocellulose membrane, Amersham Biosciences, Freiburg, Germany). X-ray films were purchased from Kodak (Kodak X-Omat, Rochester, NY, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Drug administration procedure

2.2.1. Acute homocysteine treatment

Wistar rats, aged 29 days, received a single subcutaneous injection of saline solution (control) or Hcy (0.6 µmol/g body weight). *D*,*L*-Hcy was dissolved in 0.9% NaCl solution (saline) and buffered to pH 7.4. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those found in homocystinuric patients (Mudd et al. 2001; Streck et al. 2002). Rats were euthanized by decapitation without anesthesia 1 h or 12 h after the injection; brain was quickly removed and hippocampus was dissected.

2.2.2. Chronic homocysteine treatment

D,L-Hcy were dissolved in 0.9% NaCl solution (saline) and buffered to pH 7.4. Hcy solution was administered subcutaneously twice a day from 6th to 28th days-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. Control animals received saline solution in the same volumes as those applied to Hcy treated rats. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those found in homocystinuric patients (Mudd et al. 2001; Streck et al. 2002). The rats were euthanized by decapitation without anesthesia 1 h or 12 h after the last injection; brain was quickly removed and hippocampus was dissected.

2.3 Cellular fractionation for cytosolic and nuclear NF-κB/p65 subunit

Tissue hippocampus were homogenized in 300 µL hypotonic lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM NaF, 1 mM sodium orthovanadate plus protease inhibitor cocktail). Hippocampus homogenate were than lysed with 18 µL 10% IGEPAL. The homogenate was centrifuged (14000 x *g*, 30 s, 4^{o}C), and supernatants containing the cytosolic fraction were stored at -80^{o}C. The nuclear pellet was resuspended in 200 µL ice-cold hypertonic extraction buffer (10 mM HEPES (pH 7.9), 0.40 M NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM NaF, 1

mM sodium orthovanadate, 0,25 mM EDTA, 25% glycerol plus protease inhibitor cocktail). After 40 min of intermittent mixing, extracts were centrifuged (14000 x *g*, 10 min, 4^{o}C), and supernatants containing nuclear protein were secured (Zanotto-Filho et al. 2009). Aliquots were taken for protein determination and, for electrophoresis analysis, were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris-HCl, pH 6.8.

2.4 Western blotting for *Akt, NF-κB/p65, GSK-3β and Tau* protein

For Akt, GSK-3β and *Tau* protein Western blotting analysis, hippocampus was homogenized in ice-cold lysis buffer: 20mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 Mm EDTA, 0.5% Triton X-100 and 0.1% SDS. Cytosolic and nuclear fractions were used for NF-κβ/p65 subunit according to the protocol described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). Protein samples were separated by 10% SDS-PAGE (50μg/lane of total protein) and transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% bovine serum albumin (BSA)). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05% Tween-20 (T-TBS) and then incubated overnight at 4ºC in blocking solution containing one of the following antibodies: anti-phospho Akt (Ser473) (pAkt, 1:1000; Cell Signaling Technology), anti-Akt (1:1000; Cell Signaling Technology), anti-NF-kB p65

(1:1000; Santa Cruz Biotechnology), anti-phospho GSK-3β (Ser9) (pGSK-3β, 1:1000; Cell Signaling Technology), anti-GSK-3β (1:1000; Cell Signaling Technology), anti-phospho *Tau* (Ser396) (1:1000; Cell Signaling Technology), anti-*Tau* (1:1000; Cell Signaling Technology) and anti-β-actin (1:1000, Cell Signaling Technology). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:1000. The blot was again washed twice for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). The chemioluminescence was detected using X-ray films that were scanned and analyzed using the Optiquant Software (Packard Instruments).

2.5 Protein determination

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

2.6 Statistical analysis

Data are expressed as percentage of control. Student`s *t* test was used to evaluated the different parameters. 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

Firstly we evaluated the effect of a single Hcy administration on Akt phosphorylation. This set of experiments was performed in order to evaluate when the Hcy effects starting in the hippocampus, as well as its length, given that a previous study showed that this amino acid presents a peak in the plasma at 15 min after injection, returning to baseline levels after 12 h (Streck et al., 2002). Western blotting was performed with antibodies against the active form of Akt phosphorylated at Ser 473. Fig. 1 shows that acute hyperhomocysteinemia significantly increased Akt phosphorylation at 1 h [$t(10) = 3.02$; $p<0.01$], but did not alter at 12 h [$t(9) = 0.03$; $p>0.05$] after Hcy injection.

Next, we investigated the effect of acute Hcy administration on cytosolic and nuclear fractions of NF-kB/p65 subunit. Fig. 2 shows that acute hyperhomocysteinemia significantly increased the immunocontent of cytosolic [$t(10) = 3.64$; $p<0.01$] and nuclear fraction [$t(12) = 7.57$; $p<0.001$] of NF-kB/p65 subunit at 1 h after the injection of this amino acid.

Since phosphorylated Akt (active), phosphorylates and inactivates GSK-3β (Li et al. 2000), GSK-3β was also evaluated in hippocampus of rats subjected to Hcy. We used antibodies against GSK-3β phosphorylated at Ser9. Fig. 3 shows that Hcy significantly reduced GSK-3β phosphorylation at 1 h [$t(8) = 5.27$; $p<0.001$], but did not alter at 12 h [$t(7) = 1.01$; $p>0.05$] after acute hyperhomocysteinemia.

In addition, we evaluated *Tau* phosphorylation, a protein highly phosphorylated by GSK-3β. Western blotting was performed with antibodies against the phosphorylated form of *Tau* at Ser396. Fig. 4 shows that acute hyperhomocysteinemia significantly increased *Tau* phosphorylation at 1 h [$t(10) = 3.83$; $p<0.001$] after the injection of this amino acid.

In order to mimetic homocystinuria, an inborn error of metabolism, we started chronic Hcy administration in rats on postnatal day 6, because rat neurodevelopment is equivalent to that of the human brain at birth (Clancy et al., 2007). The effect of chronic Hcy administration was also evaluated on same parameters studied after acute hyperhomocysteinemia. Fig. 5A and 5B shows that chronic hyperhomocysteinemia had no effect on Akt phosphorylation [1 h: ($t(9) = 0.25$; $p>0.05$) and 12 h: ($t(9) = 0.19$; $p>0.05$)], neither on GSK-3β phosphorylation [1 h: ($t(10) = 1.57$; $p > 0.05$) and at 12 h:($t(9) = 1.23$; $p > 0.05$)] after the last injection of Hcy.

4. Discussion

Classical homocystinuria is an inborn error of metabolism characterized by a severe deficiency of CBS activity (Mudd et al. 2001). Affected patients present tissue accumulation of Hcy and a variable symptomatology, including mental retardation, epilepsy, seizures and atherosclerosis, whose pathophysiology is poorly understood (Mudd et al. 2001).

Neuroinflammation refers to inflammatory events in the CNS that can include infiltration of cells such as macrophages and lymphocytes and an increase in inflammatory molecules that are generated centrally or peripherally and subsequently act in the CNS (Miller et al. 2009; Rivest 2009). Hcy and inflammatory alterations seem to be associated with the pathogenesis of several diseases (Welch and Loscalzo, 1998; Weiss et al. 2003; Gori et al. 2005). In this context, da Cunha et al. (2010) demonstrated that acute hyperhomocysteinemia increased cytokines (TNF-α, IL-1β, IL-6) and chemokine CCL_2 (MCP-1) in the brain of rats.

Since Akt pathway plays pivotal roles in controlling inflammation, we evaluated the effect of a single Hcy injection on Akt phosphorylation. We observed that acute hyperhomocysteinemia increased Akt phosphorylation at 1 h after injection, but did not alter this phosphorylation at 12 h after Hcy administration. Based on this first result and considering that studies have shown that after activation of Akt pathway, the phosphorylated Akt (phospho-Akt) can activate NF-κβ via enhanced phosphorylation of Iκβ (Reddy et al. 2000; Selvaraj et al., 2003; Gustin et al. 2004), we investigated the effect of a single Hcy administration on immunocontent of NF-kB/p65 subunit in cytosolic and nuclear fractions. We observed that acute hyperhomocysteinemia increased the immunocontent of NF-kB/p65 subunit in cytosolic and nuclear fractions at 1 h after acute Hcy injection in hippocampus of rats.

Activation of NF-κβ facilitates the expression and secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and MCP-1, which may elicit a pro-inflammatory status (Reddy et al. 2000; Wang et al. 2000; Gustin et al. 2004; Zhang et al. 2006). In this context, Au-Yeung et al. (2004) demonstrated that the level of serine phospho-Iκβα was elevated in cells incubated with Hcy for 15 to 60 min and returned to the basal level after 2 h of incubation. Taken together, increased phosphorylation might contribute to a significant reduction in Iκβα protein levels in Hcy-treated cells, leading to dissociation and subsequent activation of NF-κβ, a key transcription factor for pro-inflammatory immune responses (Au-Yeung et al. 2004).

Since Akt phosphorylates GSK-3β on its inhibitory serine residue, we also evaluated the effect of acute Hcy administration on GSK-3β phosphorylation. Akt is activated while GSK-3β is inhibited by phosphorylation

(Li et al. 2000). Unexpectedly, we observed that GSK3β phosphorylated was reduced at 1 h after acute injection, but was not altered at 12 h after Hcy administration. At the moment, we do not know the exact pathways involved in the decreasing of GSK-3β phosphorylation, but based on present study, this occur by mechanism independent of Akt. The GSK-3β activation could be related, for example, to oxidative stress (Hernández and Avil, 2008). Studies have show that when oxidative stress develops in conjunction with a decrease in the availability of growth factors that protect against neural oxidative stress such as insulin (or IGF-1), an activation of GSK-3β is provoked (Duarte et al., 2008). Additionally, it has been proposed that 4-hydroxynonenal, produced as a result of lipid peroxidation, could be sufficient to induce an Alzheimer's disease like pathology in mice and facilitates the formation of *Tau* aggregates ''*in vitro*'' (Perez et al., 2000; Ohsawa et al., 2008). In this context, *Tau* hyperphosphorylation occurs in mice with reduced levels of superoxide dismutase and that are therefore susceptible to an increase in oxidative damage (Melov et al., 2007). In this sense, we have previously showed that Hcy induces oxidative stress in the brain of rats, reducing antioxidant defenses, and increasing lipid peroxidation which could be related to the GSK-3β activation (decreased phosphorylation) observed in the present work (Streck et al., 2003; Matté et al., 2009a). However, more studies are needed to clarify which pathways could be acting in our experimental model.

GSK-3β enzymatic activity is involved in a variety of cellular processes including glycogen metabolism, cell-membrane-to nucleus signaling, gene transcription and survival (Jope et al. 2007). The role of GSK-3β in inflammation was first established by Martin et al. (2005) that showed that GSK-3β activity is

necessary for full stimulation of the production of several pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α, following stimulation of several types of Toll-like receptors in monocytes and peripheral blood mononuclear cells.

Other regulatory actions of GSK-3β on the innate immune system are being identified, although these have not been as extensively investigated as cytokine production (Jing et al. 2004; Lin et al. 2008). The production of nitric oxide (NO), synthesized from L-arginine by NO synthase, is also dependent on GSK-3β (Yuskaitis and Jope 2009). In this context, previous studies show that acute hyperhomocysteinemia increased nitrite levels in hippocampus of rats (da Cunha et al. 2010). In addition, dysregulation of GSK-3β has been implicated in the pathogenesis of several diseases including diabetes, Alzheimer's disease, hemorrhagic shock and sepsis (Martin et al. 2005; Dugo et al. 2006; Jope et al. 2007). Abnormal accumulation of β-amyloid proteins is a critical early stage in Alzheimer's disease neuropathology, and several studies have shown that β-amyloid production is promoted by GSK-3β and reduced by GSK-3β inhibitors (Phiel et al. 2003; Su et al. 2004).

Current data suggests that Hcy accelerates dementia by stimulating β-amyloid deposition in the brain (Mok et al., 2002; Obeid and Herrmann 2006). Recently, Matté et al. (2009b) showed that Hcy impaired short and long-term memories and reduced brain-derived neurotrophic factor levels in the hippocampus. *Tau* is an important protein in the human brain that has been implicated in memory decline, cognitive deficits and dementia (Obeid and Herrmann 2006). The effect of acute hyperhomocysteinemia on *Tau* protein phosphorylation was also evaluated. Results showed that acute hyperhomocysteinemia increased *Tau* phosphorylation at 1 h after injection.

Other reports are in agreement with our date showing a relationship between Hcy and *Tau* phosphorylation. Hcy-induced *Tau* phosphorylation has been demonstrated in the studies of Ho et al. (2002) and Chan et al. (2008), using primary cultures of neurons submitted to a prolonged treatment with Hcy or folate-deprived neuroblastoma cell line, respectively. Alternatively, Hcy-induced activation of excitatory amino acid receptors may influence the levels of *Tau* phosphorylation (Ho et al., 2002). Excitotoxicity-evoked disturbances in calcium-mediated signaling, that may alter the equilibrium in protein phosphorylation, and dephosphorylation systems are among the putative contributory factors involved in abnormal *Tau* phosphorylation (Mattson, 2003). Thus, excitotoxicity triggered by Hcy might be among the mechanisms interfering with the phosphorylation of *Tau.*

Regarding chronic treatment, results showed that Hcy did not alter Akt and GSK-3β phosphorylation by Western blotting at 1 and 12 h after the last injection of Hcy. It is reasonable speculate that the lack of effect observed in chronic administration might be a consequence from the rapid activation of these signaling pathways after the first injection; however these effects are not sustained for a long period to be observed in this chronic model. Besides, it is possible that the phosphorylation of Akt/GSK-3β probably depend on Hcy, since a previous study showed that this amino acid presents a peak in the brain at 15 min after injection, returning to baseline levels after 12 h (Streck et al. 2002). This result could also help to explain why in the acute treatment we did not find any alteration in these enzymes 12 h after Hcy injection.

In conclusion, we showed that acute hyperhomocysteinemia increases Akt phosphorylation, immunocontent of NF-κB/p65 subunit and *Tau* protein

phosphorylation, but reduces GSK-3β phosphorylation, suggesting that these alterations could be associated to neuroinflammation and/or brain dysfunction observed in some hyperhomocystinuric patients. However, many questions and intracellular mechanisms by which hyperhomocysteinemia exert these effects remain to be answered.

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Legends to Figures

Figure 1. Effect of acute hyperhomocysteinemia on Akt phosphorylation in hippocampus of rats. **(A)** Representative Western blotting showing pAkt and Akt at 1h and 12h after acute Hcy administration. **(B)** Quantification of Akt at 1h and 12h after acute Hcy administration was normalized to the total amount of Akt. Uniformity of gel loading was confirmed with β-actin as the standard. Bars represent the mean ± SD for 4-6 animals in each group. Different from control, $^{**}p<0.01$ (Student's *t*-test). Hcy: homocysteine; Akt: protein kinase B.

Figure 2. Effect of acute hyperhomocysteinemia on cytosolic and nuclear immunocontent of NF-κB/p65 subunit in hippocampus of rats. **(A)** Representative Western blotting showing cytosolic and nuclear immunocontent of NF-κB/p65 subunit at 1h after acute Hcy administration. **(B)** Quantification of cytosolic and nuclear fractions of NF-κB/p65 subunit at 1h after acute Hcy administration. Uniformity of gel loading was confirmed with β-actin as the standard. Bars represent the mean ± SD for 4-6 animals in each group. Different from control, $^{**}p<0.01$; $^{***}p<0.001$ (Student's *t*-test). Hcy: homocysteine; NF-κB: nuclear factor kappa B.

Figure 3. Effect of acute hyperhomocysteinemia on GSK-3β phosphorylation in hippocampus of rats. **(A)** Representative Western blotting showing pGSK-3β and GSK-3β at 1h and 12h after acute Hcy administration. **(B)** Quantification of GSK-3β at 1h and 12h after acute Hcy administration was normalized to the total amount of GSK3β. Uniformity of gel loading was confirmed with β-actin as

the standard. Bars represent the mean ± SD for 4-6 animals in each group. Different from control, $^{***}p<0.001$ (Student's *t*-test). Hcy: homocysteine; GSK-3β: glycogen synthase kinase-3beta.

Figure 4. Effect of acute hyperhomocysteinemia on *Tau* protein phosphorylation in hippocampus of rats. **(A)** Representative Western blotting showing p*Tau* and *Tau* at 1h after acute Hcy administration. **(B)** Quantification of *Tau* at 1h after acute Hcy administration was normalized to the total amount of *Tau*. Uniformity of gel loading was confirmed with β-actin as the standard. Bars represent the mean ± SD for 4-6 animals in each group. Different from control, $^{***}p<0.001$ (Student's *t*-test). Hcy: homocysteine; Tau: protein Tau.

Figure 5. Effect of chronic hyperhomocysteinemia on Akt and GSK-3β phosphorylation in hippocampus of rats. **(A)** Representative Western blotting showing pAkt and Akt; pGSK-3β and GSK-3β at 1h and 12h after chronic Hcy administration. **(B)** Quantification of Akt and GSK-3β at 1h and 12h after chronic Hcy administration were normalized to the total amount of Akt and GSK-3β. Uniformity of gel loading was confirmed with β-actin as the standard. Bars represent the mean ± SD for 4-6 animals in each group. Hcy: homocysteine; Akt: protein kinase B; GSK-3β: glycogen synthase kinase-3beta.

Figure 1

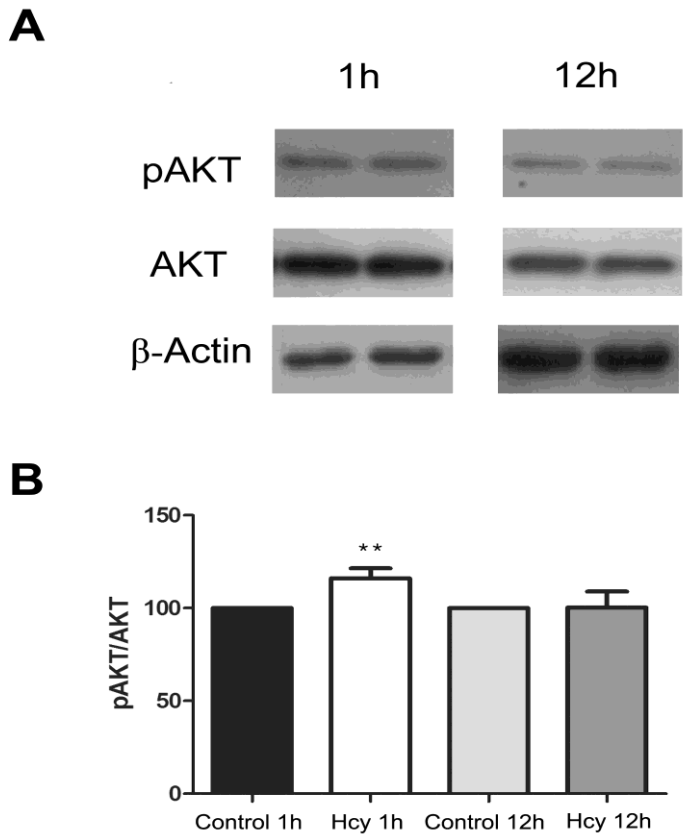


Figure 2

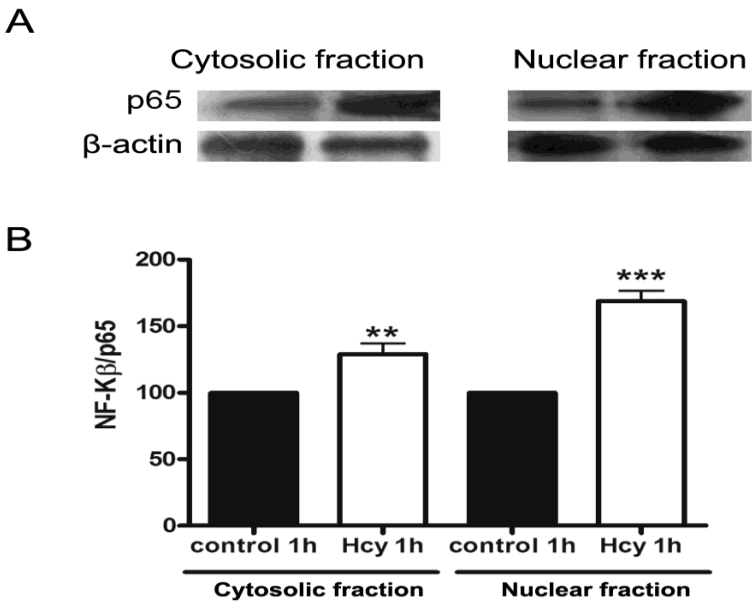


Figure 3

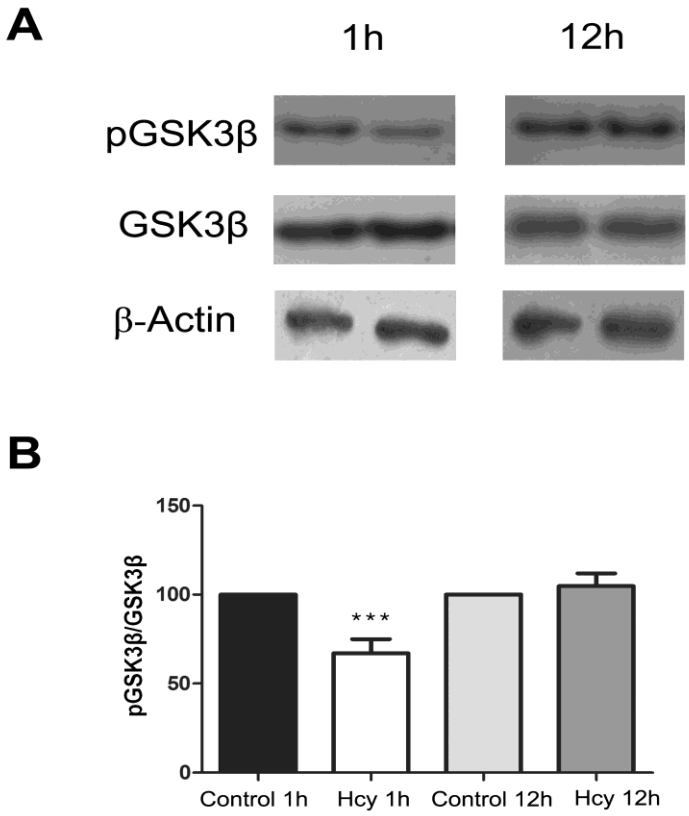


Figure 4

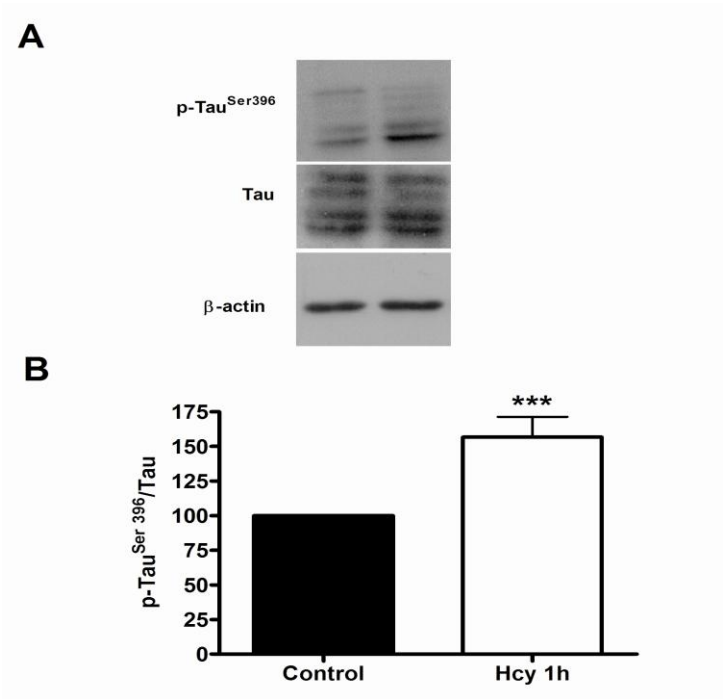
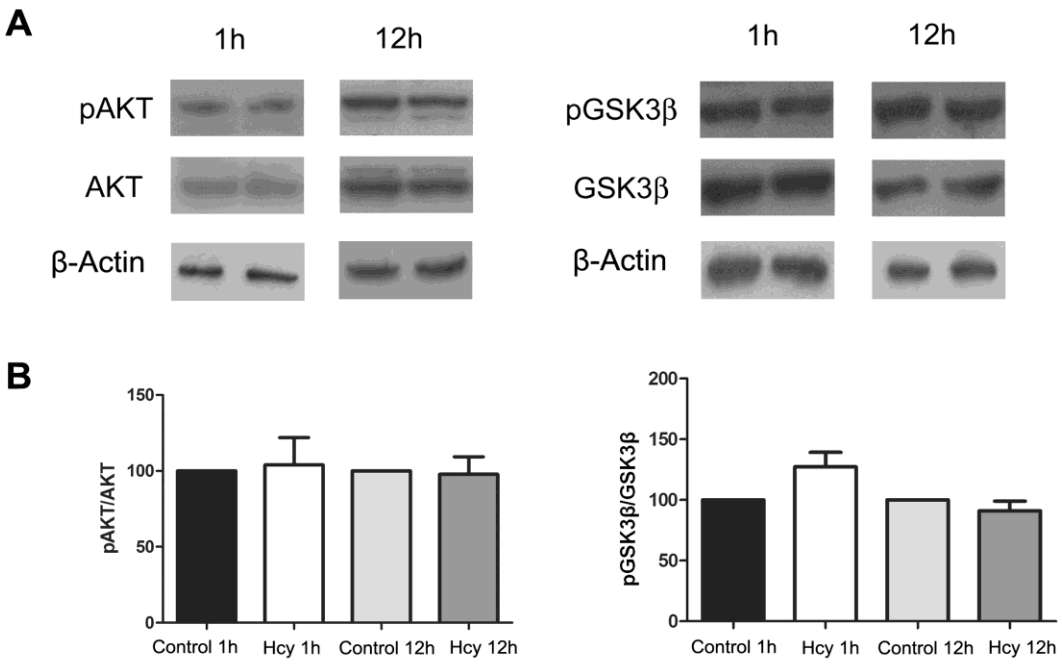


Figure 5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Control 12h
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Capítulo V

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Chronic hyperhomocysteinemia induces oxidative damage in the rat lung

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Abstract Tissue accumulation of homocysteine occurs in classical homocystinuria, a metabolic disease characterized biochemically by cystathionine β-synthase deficiency. Vascular manifestations such as myocardial infarction, cerebral thrombosis, hepatic steatosis, and pulmonary embolism are common in this disease and poorly understood. In this study, we investigated the effect of chronic hyperhomocysteinemia on some parameters of oxidative stress (thiobarbituric acid-reactive substances, protein carbonyl content, 2′,7′-dichlorofluorescein fluorescence assay, and total radical-trapping antioxidant potent) and activities of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) in the rat lung. Reduced glutathione content and glucose 6-phosphate dehydrogenase activity, as well as nitrite levels, were also evaluated. Wistar rats received daily subcutaneous injections of Hcy (0.3–0.6 μmol/g body weight) from the 6th to the 28th days-of-age and the control group received saline. One and 12 h after the last injection, rats were killed and the lungs collected. Hyperhomocysteinemia increased lipid peroxidation and oxidative damage to protein, and disrupted antioxidant defenses (enzymatic and non-enzymatic) in the lung of rats, characterizing a reliable oxidative stress. In contrast, this amino acid did not alter nitrite levels. Our findings showed a consistent profile of oxidative stress in the lung of rats, elicited by homocysteine, which could explain, at least in part, the mechanisms involved in the lung damage that is present in some homocystinuric patients.

Keywords Homocysteine · Oxidative stress · Nitrite levels · Lung

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Introduction

Hyperhomocysteinemia has been associated with several disorders that affect the central nervous system (CNS), such as epilepsy, stroke, neurodegenerative, neuropsychiatric diseases, chronic obstructive pulmonary disease [1–6], as well as inborn errors of metabolism, such as classical homocystinuria, a metabolic disease characterized biochemically by cystathionine β-synthase (CBS, EC 4.2.1.22) deficiency [7]. The most common manifestations of this disturbance are vascular complications such as myocardial infarction, cerebral thrombosis, hepatic steatosis, and pulmonary embolism [7–10].

The underlying mechanism by which Hcy exerts its toxic effects remains unexplained; however, some hypotheses have been described. It has been shown that Hcy produces reactive species [11–14], and that auto-oxidation of Hcy and other disulphides releases superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) [15, 16]. In this context, studies have suggested that free radicals play an important role in the physiopathology of acute pulmonary embolism, neurodegenerative diseases, and homocystinuria [17–21].

It is well known that oxidative stress can be defined as a serious imbalance between the production of reactive species and antioxidant defenses, and could result from diminished levels of antioxidants and/or the increased production of reactive species [22–24]. We previously showed that Hcy induces oxidative stress in the brain of

rats, reducing antioxidant defenses, and increasing lipid peroxidation [25–28]. In addition, chronic hyperhomocysteinemia decreases antioxidant defenses and total thiol content, and increases lipid peroxidation in the liver of rats, characterizing a reliable oxidative stress [29].

Since, to our knowledge, there are no studies that show any association between hyperhomocysteinemia and oxidative stress in the lung, this study evaluates the effect of chronic severe hyperhomocysteinemia, similar to those found in classical homocystinuria, on some parameters of oxidative stress, namely thiobarbituric acid-reactive substances (TBARS), protein carbonyl content, 2′,7′-dichlorofluorescein fluorescence assay (DCF) and total radical-trapping antioxidant potential (TRAP), activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). In addition, we investigated the reduced glutathione content (GSH), glucose 6-phosphate dehydrogenase (G6PD), and nitrite levels in the lung of rats subject to chronic Hcy administration.

Materials and methods

Animals and reagents

Forty Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals 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. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Chronic homocysteine treatment

D,L-Hcy were dissolved in 0.9% NaCl solution (saline) and buffered to pH 7.4. Hcy solution was administered subcutaneously twice a day from the 6th to 28th days-of-age. Hcy doses were calculated from pharmacokinetic parameters previously determined in our laboratory [30]. 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 blood of patients with severe homocystinuria (500 mM) [7, 30]. Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. The rats were killed by decapitation without anesthesia at 1 and 12 h after the last injection, and the lungs were quickly removed and kept on an ice-plate.

Tissue preparation

Lungs were homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. The homogenates were centrifuged at 750×*g* for 10 min at 4°C to separate nuclei and cell debris [31]. The pellet was discarded and the supernatant was immediately separated and used for the measurements.

Thiobarbituric acid-reactive substances

TBARS, an index of lipid peroxidation, was determined according to the method described by Ohkawa et al. [32]. For measures, tissue supernatant was mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS were determined by the absorbance at 535 nm and calculated as nmol TBARS/mg protein.

Protein carbonyl content

Oxidatively modified proteins present an enhancement of carbonyl content [33, 34]. In this study, protein carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm [35]. Results were represented as protein carbonyl content (nmol/mg protein).

2′,7′-Dichlorofluorescein fluorescence assay

Reactive species production was measured following a method described by Lebel et al. [36] based on the oxidation of 2′,7′-dichlorofluorescein (H_2DCF). H_2DCF-DA is cleaved by cellular esterases and the resultant H_2DCF is eventually oxidized by the reactive species present in samples. The latter reaction produces the fluorescent compound, DCF, which was quantified following 488 nm excitation and 525 nm emission, where results are represented by nmol DCF/mg protein.

Total radical-trapping antioxidant potential

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) thermolysis in a Perkin-Elmer Microbeta Microplate Scintillation Analyzer (PerkinElmer

Life and Analytical Sciences, Waltham, MA) [37, 38]. The time taken by the sample to maintain chemiluminescence low is directly proportional to the antioxidant capacity of the tissue. Results are represented as nmol Trolox/mg protein.

Superoxide dismutase assay

SOD activity assay is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide, which is the substrate for SOD. The inhibition of the autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed at 420 nm using the SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA) [39]. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results are reported as units/mg protein.

Catalase assay

CAT activity was assayed using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA). The method used is based on the disappearance of H_2O_2 at 240 nm [40]. One CAT unit is defined as 1 μmol of H_2O_2 consumed per minute and the specific activity is calculated as CAT units/mg protein.

Glutathione peroxidase assay

GPx activity was measured using *tert*-butyl-hydroperoxide as substrate [41]. NADPH disappearance was monitored at 340 nm using a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA). One GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as GPx units/mg protein.

Reduced glutathione content

This method is based on the reaction of GSH with the fluorophore *o*-phthalaldehyde (OPT) after deproteinizing the samples, and was measured according to Browne and Armstrong [42]. Fluorescence was then measured at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 350$ nm in a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA). A calibration curve was also performed with a commercial GSH solution, and the results were calculated as nmol GSH/mg protein.

Glucose 6-phosphate dehydrogenase assay

G6PD activity was measured according to Leong and Clark [43] in a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA). One G6PD unit is defined as 1 μmol of NADPH produced per minute and the specific activity is expressed as G6PD units/mg protein.

Nitrite assay

Nitrite levels were measured using the Griess reaction; 100 μl of tissue supernatant was mixed with 100 μl Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The absorbance was measured using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA) at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards [44].

Protein determination

Protein concentration was measured by the method of Lowry et al. [45] using bovine serum albumin as standard.

Statistical determination

Data were analyzed by the Student's *t* test for unpaired samples. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. Differences were considered statistically significant if $P < 0.05$.

Results

First, we evaluated the effect of chronic hyperhomocysteinemia on lipid and protein damage, as measured by TBARS and protein carbonyl content. Figure 1 shows that chronic hyperhomocysteinemia significantly increased TBARS and protein carbonyl content at 1 and 12 h after the last administration of Hcy (1 h [A: $t(6) = 6.11$; $P < 0.001$]; 12 h [A: $t(6) = 3.13$; $P < 0.05$]); and (1 h [B: $t(6) = 5.46$; $P < 0.001$]; 12 h [B: $t(6) = 3.72$; $P < 0.01$]), respectively.

Figure 2a demonstrates that Hcy significantly increased the levels of reactive species in lung, as indicated by DCF formed from the oxidation of H_2DCF 1 h [$t(6) = 2.84$; $P < 0.05$] and 12 h [$t(6) = 2.59$; $P < 0.05$] after chronic hyperhomocysteinemia. In addition, the status of lung non-enzymatic antioxidant defenses was evaluated. Figure 2b shows that Hcy administration did not alter the total antioxidant potential, TRAP, at 1 h [$t(6) = 0.80$; $P > 0.05$] but

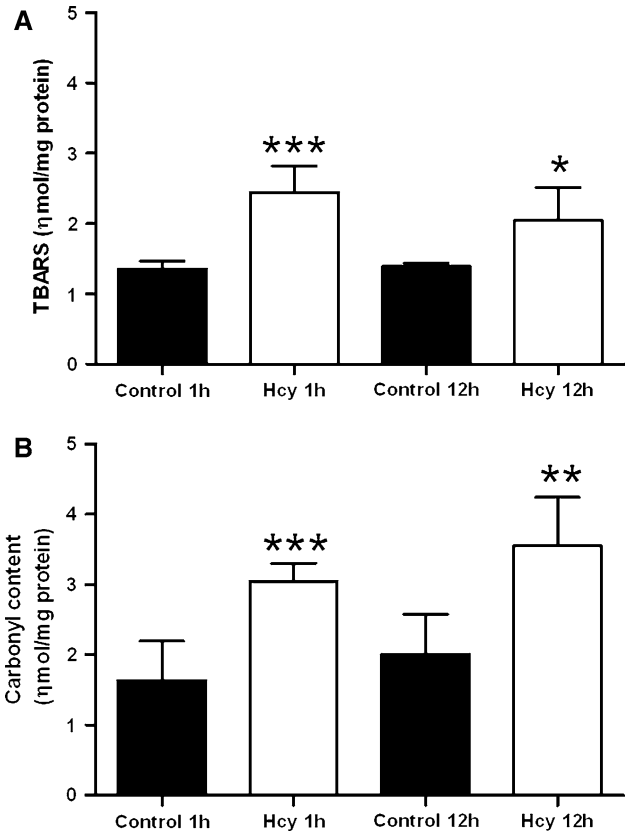

Fig. 1 Effect of chronic administration of homocysteine on thiobarbituric acid-reactive substances (**a**) and protein carbonyl content (**b**) in the lung of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t* test). *Hcy* homocysteine

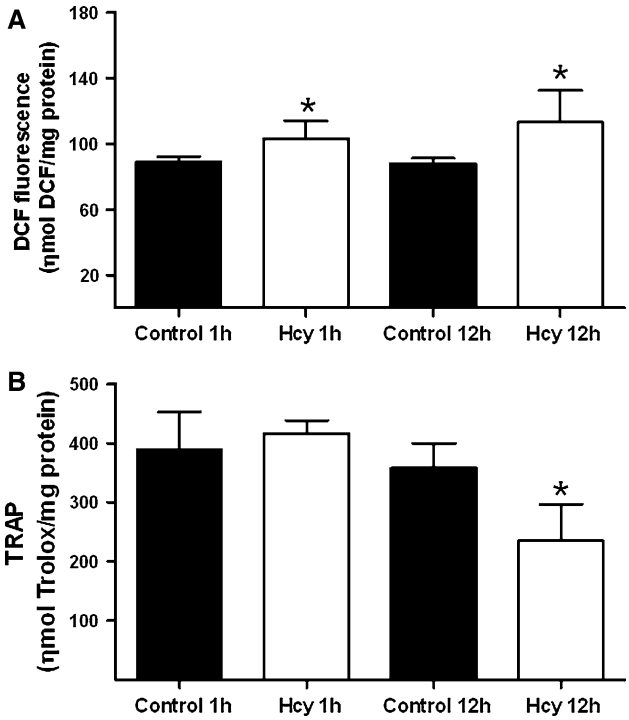

Fig. 2 Effect of chronic administration of homocysteine on 2′,7′-dichlorofluorescein fluorescence assay (**a**) and total radical-trapping antioxidant potential (**b**) in the lung of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{*}P < 0.05$ (Student's *t* test). *Hcy* homocysteine

reduced TRAP at 12 h [$t(8) = 3.13$; $P < 0.05$] in the rat lung.

Next, we evaluated the effect of chronic Hcy administration on the enzymatic antioxidant defenses (SOD, CAT, and GPx) in the lung of rats. Figure 3 shows that Hcy significantly increased the activity of SOD at 1 h [A: $t(6) = 2.77$; $P < 0.05$], but did not alter this enzyme activity at 12 h [A: $t(6) = 0.07$; $P > 0.05$] after the last injection of this amino acid. On the other hand, the activity of CAT was reduced at 1 h [B: $t(6) = 6.13$; $P < 0.001$] and 12 h [B: $t(6) = 3.10$; $P < 0.05$], when compared to the control group. The activity of GPx was not altered at 1 h [C: $t(6) = 2.08$; $P > 0.05$], but at 12 h [C: $t(6) = 3.88$; $P < 0.01$] after chronic Hcy administration the activity of this enzyme was significantly reduced in the lung of rats.

We also tested the effect of chronic hyperhomocysteinemia on GSH content and G6PD activity in the lung of rats. Figure 4 shows that chronic Hcy administration significantly reduced GSH content and G6PD activity at 1 h [A: $t(6) = 5.57$; $P < 0.001$; B: $t(6) = 4.95$; $P < 0.01$] and 12 h [A: $t(6) = 3.15$; $P < 0.05$; B: $t(6) = 3.06$; $P < 0.05$] after the last administration of Hcy. Finally, we evaluated the effect of chronic hyperhomocysteinemia on nitrite levels in the lung of rats. Figure 5 shows that nitrite levels were not altered by Hcy administration at 1 h [$t(6) = 0.61$; $P > 0.05$] and 12 h [$t(6)$, $= 3.06$; $P > 0.05$], as compared to the control group.

Discussion

Tissue levels of Hcy are severely increased in classical homocystinuria, an inborn error of metabolism characterized biochemically by CBS deficiency [7]. Clinically, affected patients present pathological manifestations in several organs, mainly in the vascular system and CNS, including mental retardation, psychiatric disturbances, seizures, thromboembolism, and cardiovascular complications [7, 46, 47]. On the other hand, data also show an association between Hcy and pulmonary disease, whose physiopathologic mechanisms are unknown [48].

It has been suggested that oxidative stress plays a central role in the pathogenesis of many pulmonary diseases [2, 49, 50]. In this context, a clinical study reported an increase of oxidative stress in patients with pulmonary embolism [21]. In addition, a preclinical study has demonstrated that CBS deficiency in mice causes lung fibrosis and that these

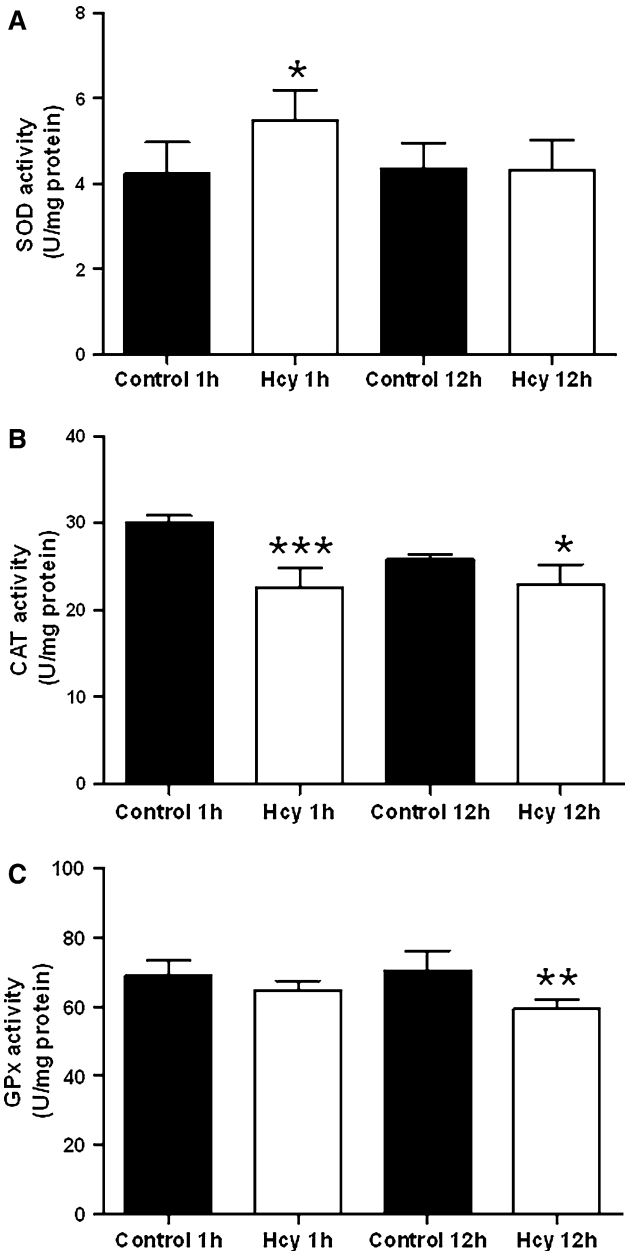

Fig. 3 Effect of chronic administration of homocysteine on superoxide dismutase (**a**), catalase (**b**), and glutathione peroxidase (**c**) in the lung of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t* test). *Hcy* homocysteine

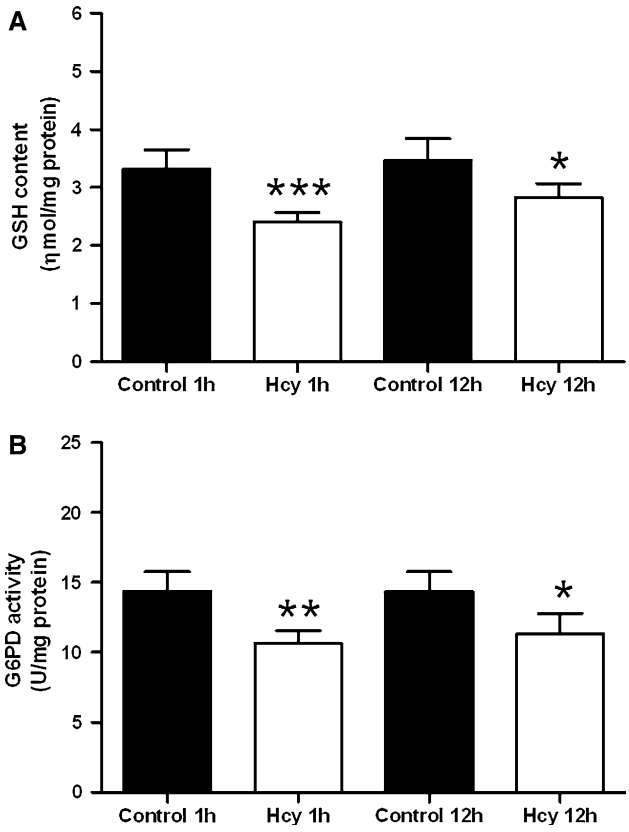

Fig. 4 Effect of chronic administration of homocysteine on reduced glutathione content (**a**) and glucose 6-phosphate dehydrogenase (**b**) in the lung of rats. Results are expressed as means ± SD for six animals per group. Different from control, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t* test). *Hcy* homocysteine

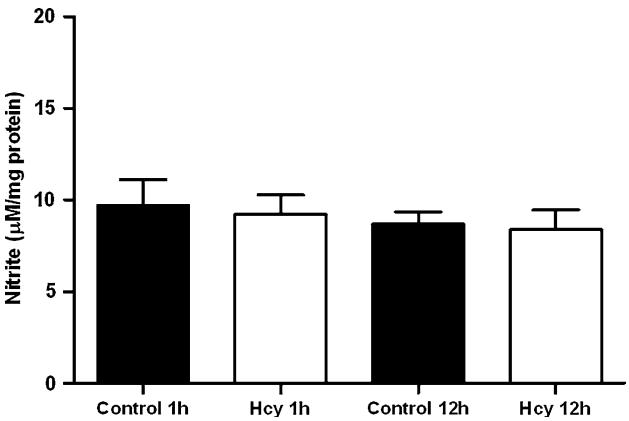

Fig. 5 Effect of chronic administration of homocysteine on nitrite levels in the lung of rats. Results are expressed as means ± SD for six animals per group. (Student's *t* test). *Hcy* homocysteine

animals present a more pronounced emphysema-like appearance than wild-type mice [51]. Therefore, the induction of lung oxidative stress induced by Hcy could potentially lead to oxidative damage and to play a central role in the pathogenesis of pulmonary damage caused by this amino acid.

Considering that, to our knowledge, no study has so far investigated the role of oxidative lung injury in rats subjected to Hcy administration; in this study, we evaluated the effect of chronic hyperhomocysteinemia on some parameters of oxidative stress. An increase was observed in the TBARS levels and protein carbonyl content at 1 and 12 h after the last injection of Hcy, suggesting that this amino acid causes lipid peroxidation (oxidative damage to

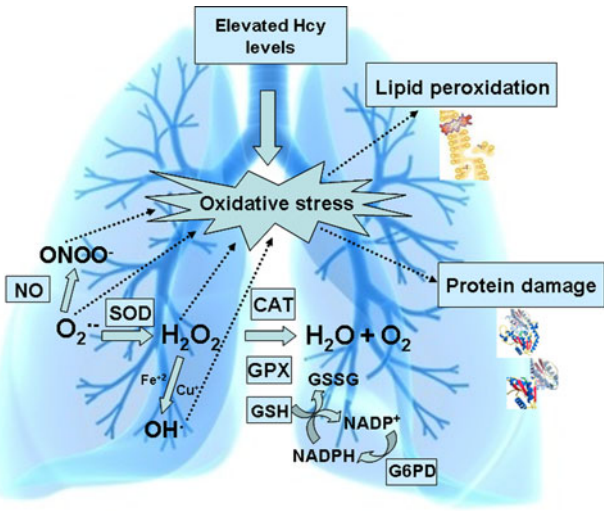

Fig. 6 Summary of the effect of homocysteine on oxidant–antioxidant status present in the lung, highlighting those processes that were quantified throughout the investigations. SOD (superoxide dismutase) CAT (catalase), GPx (glutathione peroxidase), GSH (reduced glutathione), G6PD (glucose 6-phosphate dehydrogenase), GSSG (oxidized glutathione), $NADP^+$ (oxidized nicotinamide adenine dinucleotide phosphate), NADPH (reduced nicotinamide adenine dinucleotide phosphate), *Hcy* (homocysteine)

lipids) and oxidative damage to protein. These results are in agreement with other studies showing that Hcy administration increased lipid peroxidation in the brain and liver of rats [25, 28, 29]. Also, we observed that chronic hyperhomocysteinemia significantly increased reactive species, as measured by the DCF fluorescence assay at 1 and 12 h after the last injection. It has been suggested that Hcy acts indirectly through its oxidation and the concomitant production of reactive oxygen species (ROS) [52]. In addition, it has been shown that Hcy is readily oxidized in plasma, principally as a consequence of auto-oxidation leading to the formation of metabolites such as homocysteine-mixed disulfides, and homocysteine thiolactone. During the oxidation of its sulfhydryl group, ROS are generated, and these oxygen-derived molecules are believed to account for the endothelial cytotoxicity of Hcy [53, 54].

TRAP is a useful estimate of non-enzymatic antioxidants and assesses the total potential of the main antioxidants found in lung tissue (for example, glutathione, uric acid, ascorbic acid, and α-tocopherol) [38]. In this study, we showed that chronic Hcy administration decreased TRAP at 12 h, suggesting that this amino acid causes a reduction in non-enzymatic antioxidants in the lung of rats. Furthermore, previous studies have shown that Hcy administration reduces the antioxidant potential in the parietal cortex and plasma of rats [55].

The effect of chronic hyperhomocysteinemia on the activities of antioxidant enzymes (SOD, CAT, and GPx) was also evaluated. These enzymes provide an efficient system responsible for removing ROS [22, 24]. SOD catalyses the dismutation of $O_2^{\bullet-}$, CAT catalyses the reduction of H_2O_2 and GPx detoxifies both H_2O_2 and lipid hydroperoxides [24, 56]. Results showed that Hcy administration increased SOD activity at 1 h, but not at 12 h after chronic hyperhomocysteinemia in the lung of rats. The increase in SOD activity may be a response for the enhanced levels of $O_2^{\bullet-}$. Accordingly, data from literature show that hyperhomocysteinemia increases $O_2^{\bullet-}$, by NAD(P)H oxidase activation [57]. In addition, chronic hyperhomocysteinemia reduced the activities of CAT at 1 and 12 h, and GPx at 12 h, after the last injection of Hcy. It has been previously shown that acute Hcy administration decreases CAT activity in the rat hippocampus and that vitamins E and C completely prevented this effect, indicating that the participation of oxidative stress is probably involved in the actions of Hcy [25]. In addition, the decrease in CAT and GPx activities may be explained by the fact that antioxidant enzymes are inhibited by specific ROS [58–60], which are probably formed from Hcy [61]. We believe that this imbalance between antioxidant enzymes probably alters ROS elimination [22, 24]. Therefore, it is possible that the increased SOD activity was not sufficient to completely remove the $O_2^{\bullet-}$ formed by Hcy in this experimental condition. Furthermore, the increase in $O_2^{\bullet-}$ release induced by Hcy was accompanied by an increase of H_2O_2 production [57]. It has been shown that Hcy stimulated the production of ROS and impaired the GPx-mediated detoxification of H_2O_2 in bovine aortic endothelial cells [62]. On the other hand, studies also suggest that Hcy can regulate protein turnover and gene expression, including antioxidant enzymes [63, 64].

We also observed in this study that Hcy significantly reduced GSH content and G6PD activity at 1 and 12 h after chronic Hcy administration. G6PD is the key regulatory enzyme of the pentose phosphate pathway and, as such, controls the flow of carbon through the oxidative phase of this pathway and produces reducing equivalents in the form of NADPH to meet cellular needs for reductive biosynthesis and maintenance of the cellular redox state [65]. Our studies also show that chronic administration was able to inhibit G6PD activity after Hcy injection in the lung of rats, which could promote impairment in the production of NADPH and a disruption in the cellular redox balance. This is probably in line with the observed inhibition of GPx activity at 12 h, since the activity of this enzyme depends on the regeneration of reduced glutathione by glutathione reductase, which in turn relies on NADPH that is dependent on a normal G6PD activity [66].

We also evaluated the nitrite levels in the lung of rats subjected to Hcy administration. No alterations in the levels of nitrite were observed in the lung of rats killed at 1 and 12 h after chronic hyperhomocysteinemia. Nitric oxide (NO) is a highly diffusible free radical formed by the

conversion of arginine to citrulline via a family of NO synthase (NOS) isoenzymes [67]. Furthermore, NO reacts with $O_2^{\bullet -}$ to form peroxynitrite ($ONOO^-$) [68, 69], which reacts with tyrosine residues to produce nitrotyrosine [70]. In this context, Tyagi and colleagues [17] showed that Hcy significantly increased nitrotyrosine. In addition, these authors demonstrated that increased in nitrotyrosine formation in response to Hcy did not alter the basal NOS activity. Figure 6 summarizes the oxidant–antioxidant status present in the lung, highlighting those processes that were quantified throughout the investigations.

In conclusion, chronic hyperhomocysteinemia increased lipid peroxidation, protein oxidation and DCF fluorescence assay, and altered antioxidant defenses (enzymatic and non-enzymatic). Our data showed a consistent profile of oxidative stress in the lungs of rats, elicited by Hcy, which could contribute to explain, at least in part, the mechanisms involved in the pathogenesis of lung damage in patients with classical homocystinuria.

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PARTE III

1. DISCUSSÃO

A HCU clássica é uma aminoacidopatia caracterizada bioquimicamente pela deficiência da enzima CBS, resultando em um acúmulo tecidual de Hcy que pode alcançar até 500 μM na HCU clássica. Os pacientes homocistinúricos podem apresentar um grau variado de retardo mental, distúrbios psiquiátricos, convulsões, isquemia cerebral, tromboembolismo, complicações vasculares, alterações oculares, ósseas, renais, hepáticas e pulmonares (Mudd et al., 2001). Entretanto, o exato mecanismo dos danos provocados pela HCU clássica permanece incerto, mesmo com muitos estudos realizados na tentativa de melhor compreender as alterações encontradas nessa doença. Neste contexto, Streck e cols. (2002) desenvolveram um modelo experimental de hiper-homocisteinemia severa, 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 período é similar ao de um neonato humano (Clancy et al., 2007).

Os estudos realizados pelo nosso grupo até o presente momento, utilizando o modelo experimental de hiper-homocisteinemia severa acima citado, já demonstrou que este aminoácido induz alterações no estresse oxidativo em diferentes tecidos (Wyse et al., 2002, Streck et al., 2003a; Matté et al., 2009a; Matté et al., 2009b; Kolling et al., 2011) e causa disfunção mitocondrial e depleção energética (Streck et al., 2003b), alterações comportamentais (Matté et al., 2007; Matté et al., 2009c) e excitotoxicidade, via ativação de receptores glutamatérgicos (Matté et al., 2010; Machado et al.,

2011). Entretanto, ainda não havia sido investigado nenhum parâmetro inflamatório nesse modelo experimental.

Assim, baseado em estudos prévios do nosso laboratório e considerando que o estresse oxidativo e o processo inflamatório podem estar relacionados com as alterações cerebrais e cardiovasculares encontradas nos pacientes homocistinúricos, nossos estudos iniciaram avaliando o efeito da administração aguda de Hcy sobre alguns parâmetros inflamatórios, tais como as citocinas (TNF-α, IL-1β e IL-6), a quimiocina CCL_2 (MCP-1) e os níveis de nitritos no hipocampo e no córtex cerebral de ratos. Demonstramos que a Hcy aumentou os níveis das citocinas IL-1β e IL-6 e da quimiocina CCL_2 (MCP-1), além de aumentar os níveis de nitritos no hipocampo e no córtex cerebral 15 min, 1 h e 6 h após a administração de Hcy. Em relação aos níveis de TNF-α, verificamos um aumento apenas 15 min e 1 h após a hiper-homocisteinemia aguda. Nos animais eutanaziados 12 h após a injeção de Hcy não verificamos alterações significativas nos níveis das citocinas e da quimiocina CCL_2. É possível que o aumento nos níveis de TNF-α, IL-1β, IL-6, MCP-1 e nitritos, provavelmente, dependam dos altos níveis de Hcy uma vez que esse aminoácido apresenta um pico no cérebro em 15 min após sua administração, retornando aos níveis basais após 12 h (Streck et al., 2002).

A microglia é a célula com função imune residente do SNC que quando ativada secreta uma grande variedade de citocinas pró-inflamatórias e fatores neurotóxicos, como EROS e ERNS, que contribuem para o dano neuronal observado em algumas doenças neurodegenerativas (Lerouet et al., 2002; Liu e Hong, 2003). Evidências experimentais sugerem que as EROS e a secreção de citocinas pró-inflamatórias provavelmente estejam diretamente envolvidas

na patogênese das complicações neurológicas e vasculares características dos pacientes hiper-homocisteinêmicos (Hogg, 1999).

Trabalhos realizados pelo nosso grupo demonstraram que o tratamento crônico com Hcy induz estresse oxidativo no cérebro de ratos, peroxidação lipídica e redução das defesas antioxidantes enzimáticas e não enzimáticas (Streck et al., 2003a; Matté et al., 2009a). Acreditamos que esses achados podem estar estritamente relacionados com o aumento das citocinas pró-inflamatórias e dos níveis de nitritos observados no presente estudo, uma vez que as citocinas pró-inflamatórias podem ser produzidas em resposta ao estresse oxidativo (Halliwell e Gutteridge, 2007). Outros dados corroboram com os verificados neste estudo que demonstram uma relação entre pacientes com hiper-homocisteinemia e o aumento de citocinas pró-inflamatórias (de Jong et al., 1997; Gori et al., 2005). Além disso, um recente estudo realizado por Keating e cols. (2011) demonstrou através de um modelo experimental em animais e em pacientes com HCU um aumento no perfil de várias citocinas pró-inflamatórias e de quimiocinas.

O sistema vascular também é bastante comprometido nos pacientes homocistinúricos. O tromboembolismo é a maior causa de mortalidade entre esses pacientes. Dentre os eventos resultantes do tromboembolismo, pode-se citar a oclusão de veias e artérias periféricas, que pode resultar em embolismo pulmonar, acidentes vasculares cerebrais e infarto do miocárdio (McCully, 1996; de Franchis et al., 1998; Mudd et al., 2001). Além disso, uma série de estudos tem indicado que a Hcy contribui para a progressão da aterosclerose, em parte pela inflamação vascular instalada (Wang et al., 2000; Sung et al., 2001; Wang et al., 2001). Níveis elevados de Hcy estimulam a expressão de IL-

6 em células musculares lisas de aorta de ratos (Zhang et al., 2006). Estudos *in vitro* já demonstraram que a Hcy é capaz de induzir um aumento nos níveis e na expressão de IL-8 e de MCP-1 em cultura de células endoteliais de aorta humana (Wang et al., 2000; Poddar et al., 2001; Sung et al., 2001). Neste contexto, decidimos investigar se altos níveis de Hcy podem desencadear algum efeito inflamatório sistêmico. Determinamos os níveis de TNF-α e IL-6 no soro de ratos submetidos à hiper-homocisteinemia aguda e verificamos um aumento nos níveis de TNF-α 15 min e em 1 h e um aumento nos níveis de IL-6 em 15 min, 1 h e 6 h após a administração de Hcy. Da mesma forma que os efeitos observadas no cérebro, não observamos qualquer alteração nos níveis de TNF-α e IL-6 12 h após a administração aguda deste aminoácido. O TNF-α é um mediador central da inflamação nos tecidos e induz a síntese de outras citocinas pró-inflamatórias (Lucas et al., 2006). Além disso, sua liberação pode ativar neutrófilos e células endoteliais a aumentarem a expressão de moléculas de adesão, estimulando a migração de neutrófilos para o sítio da inflamação (Argenbright e Barton, 1992), enquanto que níveis elevados de IL-6 estão intimamente relacionados ao risco de infarto do miocárdio (Lindmark et al., 2001). Há relatos na literatura de que a Hcy promove disfunção endotelial, provavelmente associada ao estresse oxidativo, o que pode levar à ativação de citocinas pró-inflamatórias no epitélio vascular (Zhang et al., 2001; Weiss, 2005; Liu et al., 2008). Assim, nossos resultados sugerem um importante papel da Hcy em nosso modelo experimental em desencadear uma resposta inflamatória sistêmica.

As proteínas de fase aguda são produzidas pelo fígado em grandes quantidades durante o processo inflamatório (Patti et al., 2002), apresentando

um papel essencial na inibição de proteases extracelulares, fibrinólise, coagulação e modulação da função de células imunes (Ridker et al., 2000). Considerando que em nosso estudo demonstramos alterações no perfil de citocinas nos animais após à hiper-homocisteinemia aguda, decidimos também avaliar os níveis de proteína C reativa (PCR) e da α_1-glicoproteína ácida no soro dos animais submetidos ao modelo agudo de hiper-homocisteinemia. Curiosamente, não verificamos alterações significativas nesses parâmetros nos diferentes tempos avaliados. Inicialmente, esperávamos encontrar um aumento nos níveis dessas proteínas de fase aguda, uma vez que em nosso estudo demonstramos um aumento nos níveis de IL-6 no soro que é uma citocina que estimula a secreção de proteínas de fase aguda pelo fígado. Entretanto, em concordância com o nosso estudo Gori e cols. (2005) também não encontraram alterações nos níveis da PCR em indivíduos idosos com hiper-homocisteinemia.

Decidimos também ,neste capítulo, investigar o perfil de leucócitos no sangue através da contagem diferencial em esfregaço sanguíneo. Os resultados mostram que o tratamento agudo com Hcy promoveu um aumento no número relativo de neutrófilos e monócitos em 15 min e 1 h, mas não observamos nenhuma alteração em 6 e 12 h após a administração aguda desse aminoácido. Relatos na literatura demonstram que um aumento nos níveis de citocinas podem induzir uma inflamação neutrofílica (Jatakanon et al., 1999) e, por outro lado os neutrófilos também podem ser importantes ativadores de citocinas como o TNF-α (Thomas et al., 1995). Interessantemente, em nosso estudo a hiper-homocisteinemia aguda provocou um aumento nos níveis de TNF-α no soro em 15 min e 1 h, e nesses mesmos

tempos observamos um quadro de neutrofilia nos animais, o que sugere uma possível relação entre o aumento nos níveis de TNF-α e o aumento no número relativo de neutrófilos. Assim, neste primeiro capítulo da tese demonstramos que o tratamento agudo com Hcy induziu uma ativação do sistema imune pelo aumento nos níveis de citocinas, quimiocinas e nitritos no hipocampo, córtex cerebral e/ou no soro dos animais, além de promover um aumento relativo no número de neutrófilos e monócitos no sangue.

Dando continuidade aos estudos do efeito da administração de Hcy sobre o processo inflamatório, o objetivo do segundo capítulo desta tese foi investigar o efeito da hiper-homocisteinemia crônica sobre os níveis de citocinas no hipocampo e no soro de ratos. Os resultados mostraram que a administração crônica de Hcy aumentou os níveis das citocinas IL-1β e IL-6 no hipocampo 1 e 12 h após a última administração deste aminoácido, enquanto que os níveis de TNF-α e MCP-1 aumentaram apenas 1 h após a hiper-homocisteinemia crônica. Embora os mecanismos precisos da ação da Hcy sobre o processo inflamatório não estejam totalmente compreendidos, nossas achados sugerem que o aumento nos níveis das citocinas poderia estar relacionado com os altos níveis cerebrais e plasmáticos deste aminoácido que atinge seu pico 15 min após sua administração (Streck et al., 2002). Estes dados corroboram com Su e cols. (2005) que demonstraram que a Hcy *in vitro* induz um aumento na expressão de citocinas inflamatórias como: TNF-α, IL-1β, IL-6, IL-8 e IL-12. É possível que as alterações no perfil das citocinas descritas neste estudo possa contribuir para o prejuízo na memória em ratos hiper-homocisteinêmicos, bem como em pacientes cujo dano cognitivo é frequentemente observado nessa doença, tendo em vista que há evidências de

que a neuroinflamação pode contribuir para algumas doenças degenerativas, bem como em algumas doenças psiquiátricas (Lucas et al., 2006).

Embora vários tipos de células do SNC (microglia, astrócitos e neurônios) são capazes de secretar citocinas, células derivadas da periferia também contribuem para o desenvolvimento de lesões e inflamação no cérebro, através de alterações na barreira hemato-encefálica que permitem a migração transendotelial de células do sistema imune periférico (Giulian et al., 1989). Assim, dando continuidade a este estudo, verificamos que a administração crônica de Hcy aumentou os níveis de TNF-α, IL-1β, IL-6 e MCP-1 no soro de ratos eutanaziados 1 e 12 h após a hiper-homocisteinemia crônica.

Dados na literatura demonstram que a Hcy pode contribuir para a progressão da aterosclerose que pode principalmente, ser uma consequência da inflamação vascular causada por esse aminoácido (Wang et al., 2000; Sung et al., 2001). Na lesão induzida pela Hcy, as células endoteliais são ativadas sendo capazes de produzir várias moléculas de adesão e quimiocinas, tais como, VCAM-1, ICAM-1, E-selectina, P-selectina, integrina-β1, IL-8 que participam da reação inflamatória na parede arterial (Poddar et al., 2001; Hofmann et al., 2001; Sung et al., 2002, Postea et al., 2006). A Hcy também estimula a liberação de TNF-α e aumenta a adesão de monócitos humanos nas células endoteliais, além de estimular a secreção de outras citocinas pró-inflamatórias (Dalal et al., 2003; Su et al., 2005). Estes resultados sugerem que a Hcy pode contribuir para a iniciação e a progressão da doença vascular, através da secreção de citocinas e pela ativação de monócitos que amplificam a resposta inflamatória.

Também observamos um aumento nos níveis de nitritos 1 e 12 h no hipocampo de ratos após a hiper-homocisteinemia crônica. O NO é produzido por um grupo de enzimas chamadas de NOS que convertem o aminoácido arginina em citrulina e NO (Alderton et al., 2001). O NO regula uma variedade de processos fisiológicos apresentando um importante papel no sistema cardiovascular, na transmissão de estímulos nervosos, na fisiologia pulmonar, na coagulação sanguinea, na angiogênese, na defesa celular, entre outros (Cooke e Losordo, 2002). Durante a inflamação, os níveis de NO aumentam consideravelmente pela ativação da iNOS por citocinas e células inflamatórias (Pacher et al., 2007). Neste contexto, Welch e cols. (1998) demonstraram em seu estudo que a Hcy induz a síntese de NO em células da musculatura lisa após ativação da iNOS que foi demonstrada ser dependente da ativação NF-κβ. Além disso, resultados já investigados nesta tese demonstram um aumento nos níveis de nitritos no hipocampo e no córtex cerebral de animais submetidos ao modelo agudo de hiper-homocisteinemia (da Cunha et al., 2010).

Por outro lado, encontramos resultados diferentes no soro. Verificamos que a Hcy diminuiu os níveis de nitritos 1 h após a hiper-homocisteinemia crônica, mas não alterou esse parâmetro em 12 h. Em condições normais, o NO desempenha um papel na detoxificação da Hcy através da formação de S-nitroso-homocisteína (Stamler et al., 1993). Além disso, a exposição crônica à Hcy aumenta a formação de $O2^{\bullet-}$, que pode reagir com o NO e produzir outro radical livre considerado um potente agente oxidante, o $ONOO^-$ (Zhang et al., 2000; Tyagi et al., 2005). Neste contexto, Givvimani e cols. (2011) demonstraram que a Hcy estimula a geração de $ONOO^-$ em mitocôndrias extraídas do tecido cardíaco. Acreditamos que a diminuição da

biodisponibilidade do NO pode estar reduzida devido à geração de EROS causadas pela auto-oxidação da Hcy, já bem estabelecida na literatura. Outros estudos verificaram que o aumento do estresse oxidativo vascular na hiper-homocisteinemia não só pode levar à diminuição da biodisponibilidade do NO, mas também ativar vias de sinalização redox-sensíveis que induzem a um estado pró-inflamatório (Woo et al., 2003; Postea et al., 2006).

As prostaglandinas são membros da família dos eicosanóides e são sintetizados a partir do ácido araquidônico, através da ação de enzimas ciclooxigenase (COX) (Berenbaum, 2000). A PGE_2 é o prostanóide mais abundante no corpo humano (Serhan e Levy, 2003), e tem sido considerada a prostaglandina principal na inflamação aguda, em doenças como a artrite reumatóide e a osteoartrite (Bombardier et al., 1981; Amin et al., 1996). Verificamos que a administração crônica de Hcy aumentou a PGE_2 no hipocampo 1 e 12 h após a última injeção de Hcy. Por outro lado, no soro, demonstramos que Hcy aumentou os níveis de PGE_2 apenas 1 h após a última administração de Hcy. Relatos na literatura indicam que a iNOS pode se ligar especificamente na COX-2, resultando em um aumento na atividade da COX-2 com o consequente aumento na produção de PGE_2 (Kim et al., 2005). Assim, acreditamos que os efeitos encontrados em relação aos níveis de PGE_2 no hipocampo podem ter sido encontrados pelo aumento nos níveis de nitritos observados em nosso estudo.

Avaliamos também neste capítulo o imunoconteúdo das frações citoplasmática e nuclear da subunidade p65 do NF-κB, uma vez que este fator de transcrição está intimamente relacionado com a resposta inflamatória. Verificamos que a hiper-homocisteinemia crônica aumentou significativamente

o imunoconteúdo de ambas as frações da subunidade p65 do NF-κB 1 h após a última injeção de Hcy, mas não observamos alterações no imunoconteúdo 12 h após à administração crônica desse aminoácido.

O aumento do estresse oxidativo na hiper-homocisteinemia tem sido relacionado com a ativação de vias de sinalização pró-inflamatórias, incluindo a via do NF-κβ (Au-Yeung et al., 2004). Neste contexto, foi demonstrado que a Hcy estimula a expressão de ICAM-1 e de TNF-α em células endoteliais, mediante ativação do NF-κβ por uma via dependente da proteína cinase ativada por mitógeno (MAPK) (Bai et al., 2007). O NF-κβ também pode ser ativado através de mecanismos de sinalização mediados pela proteína cinase C (PKC) que parece ser estimulada pela Hcy (Wang et al., 2000). Por outro lado, estudos recentes já demonstraram o envolvimento de altos níveis de Hcy com o estresse oxidativo (Streck et al., 2003a; Matté et al., 2009a), e que elevados níveis de EROS podem ativar a via do NF-κβ (Li e Karin, 1999). Neste contexto, Matté e cols. (2009a) demonstraram em seu estudo que a administração crônica de Hcy promoveu dano ao DNA, avaliado pelo teste do cometa, e diminuiu as defesas antioxidantes (enzimáticas e não-enzimáticas) no córtex parietal, sangue e/ou plasma de ratos. Em resumo, a via de sinalização do NF-κβ pode ser um ponto de convergência pelo qual diferentes fatores podem estar envolvidos no processo inflamatório como observado em nosso modelo experimental de hiper-homocisteinemia severa.

A atividade colinérgica é controlada principalmente pela enzima AChE que hidrolisa rapidamente a acetilcolina nas sinapses colinérgicas e junção neuromuscular finalizando a transmissão colinérgica. Considerando o efeito de supressão da resposta inflamatória pela acetilcolina, acredita-se que a

atividade AChE possa ser considerada um regulador inflamatório (Borovikova et al., 2000). Foi demonstrado que a administração de inibidores de AChE reduz os níveis séricos de citocinas pró-inflamatórias e melhora a sobrevida em um modelo experimental de sepse em camundongo (Hofer et al., 2008); outro estudo mostrou que a acetilcolina diminuiu significativamente a liberação de citocinas *in vitro*, como o TNF-α, IL-1β, IL-6 e IL-18 em cultura de macrófagos humanos estimulados com LPS (Borovikova et al., 2000). Com base nesse achado e, finalizando esse capítulo da tese, decidimos avaliar o efeito da administração crônica de Hcy sobre a atividade da AChE. Verificamos que a atividade desta enzima aumentou no hipocampo 1 h após a última injeção de Hcy, mas não alterou 12 h após a hiper-homocisteinemia crônica. Considerando o papel da acetilcolina em inibir a liberação de citocinas pró-inflamatórias (Borovikova et al., 2000; Pollak et al., 2005), podemos especular que este aumento na atividade da AChE pode causar um prejuízo na capacidade da acetilcolina na regulação do processo inflamatório, o que pode estar relacionado com o aumento nos níveis das citocinas demonstradas até o momento. Portanto, nesse segundo capítulo da tese demonstramos que o tratamento crônico com a Hcy também foi capaz de induzir uma ativação do sistema imune no hipocampo dos ratos, demonstrado pelo aumento nos níveis de citocinas, da quimiocina CCL_2, dos níveis de nitritos, do imunoconteúdo das frações citoplasmática e nuclear da subunidade p65 do NF-κB, além do aumento na atividade da acetilcolinesterase, entretanto no soro verificamos um aumento nos níveis das citocinas, mas uma diminuição nos níveis de nitritos no soro dos animais após a hiper-homocisteinemia crônica. Um resumo dos

principais resultados observados no cérebro e no sangue encontram-se nos Anexos 1 e 2.

A Hcy parece ter diferentes efeitos tóxicos sobre o sistema vascular. Neste contexto, estudos *in vitro* buscam identificar os possíveis mecanismos pelos quais a Hcy pode estar atuando, incluindo lesão de células endoteliais (Wall et al., 1980), aumento da expressão de moléculas de adesão (Silverman et al., 2002), redução na produção de prostaciclina (Wang et al., 1993), redução na síntese de NO (Stamler et al., 1993) e do fator de relaxamento derivado do endotélio (Weiss et al., 2002), indução de trombose pela ativação do fator V (Rodgers e Kane, 1986), bem como o desenvolvimento de estresse oxidativo (Mujumdar et al., 2001; Wyse et al., 2002; Streck et al., 2003a). Em conjunto, esses dados demonstram os efeitos multifatoriais da Hcy na doença vascular. Assim, nosso próximo objetivo foi investigar o efeito da administração aguda de Hcy sobre parâmetros do sistema da coagulação sanguínea. As plaquetas exercem uma função fundamental na hemostasia e na reparação de feridas (Biousse, 2003). Assim, inicialmente demonstramos um aumento na contagem de plaquetas 15 min e 1 h após a injeção deste aminoácido, entretanto 6 e 12 h após a administração de Hcy não observamos alterações significativas neste parâmetro. Acreditamos que o aumento na contagem de plaquetas esteja relacionado com os altos níveis plasmáticos de Hcy que em 15 min encontram-se no pico plasmático deste aminoácido (Streck et al., 2002).

Anormalidades na adesão e na agregação plaquetária têm sido descritas em alguns pacientes homocistinúricos (Mudd et al., 2001). Além disso, um estudo recente demonstrou que o tratamento com Hcy aumenta a agregação plaquetária induzida pelo ADP (Zanin et al., 2010). Dando continuidade a esse

capítulo, determinamos o tempo de protrombina (TP) e o tempo de tromboplastina parcial ativada (KTTP). Os resultados obtidos demonstram que a Hcy promove uma diminuição no TP e KTTP no plasma 15 min e 1h, mas não em 6 e 12 h após a administração de Hcy, indicando que altos níveis deste aminoácido na circulação promovem um estado de hipercoagulabilidade no plasma desses animais.

Anormalidades das vias extrínsecas e intrínsecas da coagulação podem ser detectadas pela determinação de parâmetros da rotina laboratorial como o TP e o KTTP (Kurata e Horii, 2004). O TP é utilizado como teste de triagem para avaliação dos fatores da via extrínseca: VII, X, V e II (Quick et al., 1935) e o KTTP para avaliação dos fatores da via intrínseca: XII, XI, IX, VII, X, V e II (Proctor e Rapaport, 1961). Já foi demonstrado que ratos hiper-homocisteinêmicos apresentam um aumento no plasma do fator VIII (Ebbesen e Ingerslev, 2005). Este fator participa da ativação do fator V e seus níveis quando elevados estão associados ao risco de tromboembolismo venoso (Koster et al., 1995; O`Donnell et al., 2000; Bombeli et al., 2002). Neste contexto, Lijfering e cols. (2007) demonstraram em seu estudo que o aumento do risco de doenças cardiovasculares na hiper-homocisteinemia está relacionada principalmente aos elevados níveis de factor VIII. Outros mecanismos que podem contribuir para o estado de hipercoagulabilidade na hiper-homocisteinemia incluem a ativação dos fatores V e XII (Rodgers e Kane, 1986). Por outro lado, Maclean e cols. (2010) não observaram alterações significativas nos parâmetros de coagulação em um modelo de HCU clássica pela deficiência da enzima CBS em camundongo.

O fibrinogênio é uma glicoproteína que é sintetizada no fígado e encontrada no plasma e nos grânulos das plaquetas. Essa glicoproteína desempenha um papel central na hemostasia, sendo responsável pela formação da rede de fibrina (Roberts et al., 2001). Além disso, estudos relatam que elevações nos níveis de fibrinogênio estão associados com um risco aumentado de doença trombótica (Hantgan et al., 2001). Em relação aos níveis de fibrinogênio, demonstramos que a hiper-homocisteinemia promoveu um aumento nos níveis de fibrinogênio no plasma 15 min e 1 h após a administração aguda deste aminoácido. Um estudo realizado em coelhos submetidos à administração crônica de Hcy demonstrou que os animais apresentaram uma disfibrinogenemia, desencadeando alterações estruturais na formação e na estrutura da rede de fibrina (Sauls et al., 2003).

A indução do estresse oxidativo também favorece um estado pró-coagulante, influenciando a função de importantes mediadores da fibrinólise (Zhang et al., 2002). Além do fibrinogênio ser um importante fator da coagulação relacionado com hipercoagulabilidade (Zhang et al., 2002), o fibrinogênio é também conhecido como uma proteína de fase aguda da inflamação (Rekka e Chrysselis, 2002). Assim, o aumento nos níveis de fibrinogênio pode estar associado com a inflamação desencadeada pela Hcy, como já evidenciado nesta tese pelo aumento nos níveis de diferentes citocinas como o TNF-α e a IL-6 (da Cunha et al., 2010).

Considerando que o NO desempenha um papel importante em vários processos fisiológicos e tem sido implicada em uma série de doenças vasculares (de Groote et al., 1996), seguimos determinando os níveis de nitritos no plasma dos ratos submetidos à administração aguda de Hcy.

Verificamos uma diminuição nos níveis de nitritos 15 min e 1 h, mas não observamos alterações significativas em 6 ou 12 h após a administração de Hcy. Sabe-se que a liberação de NO pelo endotélio regula o fluxo sanguíneo e a agregação plaquetária e que alterações em sua liberação podem estar relacionadas com a disfunção endotelial, favorecendo a formação de trombos e a aterosclerose (Upchurch et al., 1997a). Existem controvérsias quanto aos efeitos do NO na hiper-homocisteinemia. É descrito na literatura que a hiper-homocisteinemia pode promover um aumento nos níveis de NO (Nishio e Watanabe, 1997) ou uma diminuição em seus níveis (Tyagi, 1998). Acredita-se que a Hcy aumenta a expressão de iNOS por macrófagos promovendo dano às células vasculares, mas também é capaz de diminuir a expressão de eNOS pelas células endoteliais sendo considerado um dos responsáveis pela disfunção endotelial encontrada nesta doença (Zhang et al., 2000).

Um dos principais responsáveis pela diminuição nos níveis de NO em pacientes hiper-homocisteinêmicos é provavelmente a geração de EROS (Karolczak e Olas, 2009). Fischer e cols. (2003) demonstraram que o $ONOO^-$ que é produzido pela reação do NO com $O_2^{\bullet-}$ é um potente agente oxidante responsável por iniciar o processo de lipoperoxidação por promover a oxidação e nitração de proteínas, sendo que este processo de nitração pode ser responsáveis pela diminuição nos níveis de NO, uma vez que a Hcy reage com NO para formar S-nitroso-homocisteína (Ignarro e Gruetter, 1980).

São conhecidos os efeitos da Hcy em promover danos celulares através da indução de estresse oxidativo (Mujumdar et al., 2001; Wyse et al., 2002; Streck et al., 2003a), finalizamos esse terceiro capítulo analisando o efeito da hiper-homocisteinemia aguda sobre os níveis de TBARS, índice de

lipoperoxidação, no plasma de ratos. Demonstramos um aumento nos níveis de TBARS 15 min e 1h após a injeção de Hcy. A Hcy é facilmente oxidada no plasma, principalmente como conseqüência de sua auto-oxidação, levando à formação de homocisteína, dissulfetos mistos de homocisteína e homocisteína tiolactona. Durante a oxidação de seu grupo sulfidrila, espécies reativas podem ser geradas, tais como o $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, e sabe-se que essas espécies podem ter importantes efeitos nocivos (Tyagi, 1998; Nishio e Watanabe 1997; Welch et al., 1998), podendo ser responsáveis por iniciar a lipoperoxidação (Heinecke, 1988). Além disso, foi demonstrado que a Hcy induz um consistente estresse oxidativo no hipocampo, córtex parietal e fígado de ratos e reduz as defesas antioxidantes enzimáticas e não-enzimáticas (Loscalzo, 1996; Streck et al., 2003a; Matté et al., 2009a; Matté et al., 2009b). Buscando compreender melhor os efeitos da administração aguda de Hcy sobre o estresse oxidativo avaliamos a atividade das defesas antioxidantes (SOD, CAT e GPx) em eritrócitos dos ratos. Nossos resultados demonstram que os animais submetidos à hiper-homocisteinemia aguda apresentam um desequilíbrio na relação das enzimas SOD e CAT, expressa pela diminuição na atividade da SOD e pelo aumento na atividade da CAT em eritrócitos de ratos. Sabe-se que a SOD e a CAT apresentam funções importantes na remoção de EROS, catalisando a dismutação do $O_2^{\bullet-}$, seguido pela conversão do H_2O_2 em H_2O e O_2 (Halliwell e Gutteridge, 2007). Acreditamos que a diminuição da SOD possa ter sido resultado de um acúmulo de $O_2^{\bullet-}$ neste estudo, e este achado pode ter sido responsável em estimular o aumento da atividade da enzima seguinte nessa via de eliminação de EROS, a CAT. Também verificamos uma diminuição na atividade da GPx 15 min após a última administração de Hcy, o que

compromete a eficiência de detoxificação das EROS que pode levar à dano oxidativo a biomoléculas (Halliwell e Gutteridge, 2007). Além disso, a Hcy pode diminuir a biodisponibilidade do NO por gerar H_2O_2 e promover a diminuição da atividade da GPx, sendo fatores responsáveis pelos efeitos tóxicos da Hcy sobre o endotélio (Upchurch et al., 1997a). Baseado nos resultados desse capítulo sugerimos que a hiper-homocisteinemia aguda promove um estado de hipercoagulabilidade e induz estresse oxidativo no sangue dos animais, o que pode estar relacionado com a disfunção vascular presente em alguns pacientes hiper-homocisteinêmicos. Um resumo dos principais resultados observados no sangue encontram-se no Anexo 2.

A via da Akt desempenha um papel crucial no controle da neuroinflamação. Nesta etapa do trabalho, avaliamos o efeito da administração aguda de Hcy na fosforilação da Akt. Observamos que a hiper-homocisteinemia aguda aumentou a fosforilação da Akt no hipocampo 1 h após a injeção de Hcy, mas não alterou este parâmetro em 12 h. A expressão e secreção de citocinas está intimamente relacionada com a via da Akt (Selvaraj et al., 2003). Estudos têm demonstrado que a fosforilação da Akt pode ativar a via do NF-κβ através da fosforilação da Iκβ (principalmente Iκβα), favorecendo a translocação do NF-κβ do citoplasma para o núcleo (Reddy et al., 2000; Gustin et al., 2004). No núcleo, o NF-κβ facilita a expressão e secreção de citocinas pró-inflamatórias, como TNF-α e IL-1β (Reddy et al., 2000; Gustin et al., 2004). Além disso, várias linhas de evidências indicam que a Hcy induz a expressão de IL-6 e MCP-1 via ativação do NF-κβ (Wang e Siow, 2000; Zhang et al., 2006).

Também investigamos o efeito da hiper-homocisteinemia aguda sobre imunoconteúdo das frações citoplasmática e nuclear da subunidade p65 do NF-kB. A administração aguda de Hcy aumentou o imunoconteúdo de ambas as frações da subunidade p65 do NF-kB 1 h após a hiper-homocisteinemia aguda no hipocampo de ratos. Neste contexto, Au-Yeung e cols. (2004) demonstraram que células incubadas com Hcy por 15 a 60 min aumentaram a fosforilação da Iκβα, sendo que seu nível retornou ao basal após 2 h de incubação, o que pode ser explicado pela rápida ativação dessas vias de sinalização. Esse aumento da fosforilação da Iκβα leva a sua dissociação do NF-κβ que ativado torna-se um importante fator de transcrição.

Posteriormente, investigamos o efeito da administração aguda de Hcy sobre a fosforilação da GSK-3β, que representa um dos substratos da Akt. Demonstramos uma redução na fosforilação da GSK-3β 1 h após a injeção aguda deste aminoácido, por outro lado não observamos alteração neste parâmetro em 12 h. Uma vez que a Akt fosforila a GSK-3β em seu resíduo de Ser-9, também avaliamos o efeito da administração aguda de Hcy sobre a fosforilação GSK-3β. A Akt quando fosforilada encontra-se em sua forma ativa, enquanto que a GSK-3β quando fosforilada está em sua forma inativa (Li et al., 2000). Inesperadamente, observamos uma diminuição na fosforilação da GSK-3β 1 h após a injeção de Hcy, entretanto 12 h após a administração aguda de Hcy não verificamos alteração neste parâmetro. Até o momento, não sabemos o exato mecanismo envolvido na diminuição da fosforilação da GSK-3β, mas com base no presente estudo, esse mecanismo parece ser independente de Akt. A ativação da GSK-3β pode estar relacionada ao estresse oxidativo (Hernández e Avil, 2008). Estudos demonstram que o estresse oxidativo em

conjunto com uma diminuição na disponibilidade de fatores de crescimento que protegem contra o estresse oxidativo neural como a insulina, favorecem uma ativação da GSK-3β (Duarte et al., 2008). Além disso, um estudo demonstrou que a presença do 4-hidroxinonenal (4-HNE), produto da lipoperoxidação, pode induzir ao desenvolvimento da doença de Alzheimer em camundongos por facilitar a formação de agregados da proteína *Tau* (Perez et al., 2000; Ohsawa et al., 2008). Neste contexto, outro estudo demonstrou que a hiperfosforilação da proteína *Tau* ocorre em animais com níveis reduzidos de SOD, tornando-os suscetíveis ao dano oxidativo (Melov et al., 2007). Neste sentido, nosso grupo de pesquisa já demonstrou que a Hcy induz estresse oxidativo no cérebro de ratos, o que pode estar relacionado à ativação da GSK-3β como observada neste capítulo da tese (Streck et al., 2003a; Matté et al., 2009a). Entretanto, mais estudos são necessários para esclarecer quais mecanismos poderiam estar atuando em nosso modelo experimental.

A atividade enzimática da GSK-3β está envolvida em uma variedade de processos celulares, incluindo o metabolismo de glicogênio, sinalização celular, proteínas estruturais, fatores de transcrição e na sobrevivência celular (Jope et al., 2007). O papel da GSK-3β na inflamação foi inicialmente descrito por Martin e cols. (2005) que demonstraram que a atividade da GSK-3β é necessária para a secreção de diversas citocinas pró-inflamatórias, como TNF-α IL-1β e IL-6.

Outras ações regulatórias da GSK-3β sobre o sistema imune inato estão sendo identificados (Jing et al., 2004; Lin et al., 2008). A produção de NO, também é dependente de GSK-3β (Yuskaitis e Jope, 2009). Neste contexto, já demonstramos que a hiper-homocisteinemia aguda aumenta os níveis de nitritos no hipocampo de ratos (da Cunha et al., 2010).

A desregulação da GSK-3β tem sido implicada na patogênese de várias doenças, incluindo diabetes, Doença de Alzheimer, choque hemorrágico e sepse (Martin et al., 2005; Dugo et al., 2006; Jope et al., 2007). Na doença de Alzheimer as placas senis extracelulares, são formadas através do acúmulo e agregação do peptídeo β-amilóide, formando filamentos cercados por neuritos distróficos, microglia ativada e astrócitos reativos, enquanto que os emaranhados neurofibrilares são formados pela deposição da proteína *Tau* que encontra-se hiperfosforilada, acumulando intracelularmente na forma de filamentos emaranhados helicoidais pareados (Phiel et al., 2003).

Vários estudos têm demonstrado que a fosforilação da proteína *Tau* é promovida, principalmente pela GSK-3β (Phiel et al., 2003; Su et al., 2004). Assim, o efeito da hiper-homocisteinemia aguda sobre fosforilação da proteína *Tau* também foi investigado. Os resultados demonstram que a hiper-homocisteinemia aguda aumentou a fosforilação da proteína *Tau* 1 h após a administração de Hcy. Outros relatos na literatura estão de acordo com os demonstrados nesta tese. Ho e cols. (2002) e Chan e cols. (2008) demonstraram em seus estudos um aumento na fosforilação da proteína *Tau* em culturas primárias de neurônios submetidos a um tratamento prolongado com Hcy ou em uma linhagem de neuroblastoma com privação de folato.

Além disso, dados sugerem que a Hcy acelera a demência estimulando a deposição de proteína β-amilóide no cérebro (Obeid e Herrmann, 2006). A proteína *Tau* é uma importante proteína cerebral que tem sido implicada no declínio da memória e no déficit cognitivo (Obeid e Herrmann, 2006). Neste contexto, recentemente Matté e cols. (2009c) demonstraram que a Hcy

compromete a memória de curta e longa duração e reduz o fator neurotrófico derivado do encéfalo (BDNF) no hipocampo de ratos.

Também investigamos, neste estudo, o efeito da hiper-homocisteinemia crônica sobre as vias de sinalização da Akt e da GSK-3β. Não verificamos nenhuma alteração significativa na fosforilação da Akt e GSK-3β 1 e 12 h após a última administração de Hcy. Podemos especular que o efeito observado na hiper-homocisteinemia crônica possa ser resultado de um mecanismo de adaptação do organismo do animal, uma vez que encontramos alterações apenas no modelo agudo de hiper-homocisteinemia, provavelmente pela rápida ativação destas vias de sinalização. Além disso, é possível que a fosforilação da Akt e da GSK-3β dependam de altos níveis de Hcy, uma vez que este aminoácido retorna aos níveis basais 12 h após sua administração (Streck et al. 2002), o que pode ajudar a explicar o fato de que no tratamento agudo não encontramos alterações nestas vias 12 h após a administração de Hcy.

Concluimos neste quarto capítulo que a hiper-homocisteinemia aguda aumenta a fosforilação da Akt, o imunoconteúdo das frações citoplasmática e nuclear da subunidade p65 do NF-κB e a fosforilação da proteínas *Tau*, mas reduziu a fosforilação da GSK-3β, sugerindo que estas alterações podem estar associadas com a neuroinflamação e/ou com a disfunção cerebral observada em alguns pacientes homocistinúricos. Um resumo dos principais resultados observados no cérebro encontram-se no Anexo 1.

Sabe-se que além de todas as alterações neurológicas e cardiovaculares, alguns pacientes homocistinúricos podem apresentar tromboembolismo pulmonar (Dikshit et al., 1989; Andersson et al., 2001; Hamelet et al., 2007). Além disso, um estudo pré-clínico demonstrou que

camundongos com deficiência de CBS desenvolveram fibrose pulmonar e um pronunciado enfisema (Ryrfeldt et al., 1992). Considerando que até o momento nenhum estudo investigou o papel do estresse oxidativo no tecido pulmonar de ratos submetidos ao modelo crônico de hiper-homocisteinemia, no quinto e último capítulo desta tese, avaliamos o efeito da administração crônica de Hcy sobre alguns parâmetros de estresse oxidativo neste órgão.

Uma das consequências do aumento da produção de EROS é o dano a biomoléculas, tais como proteínas e lipídios. Demonstramos um aumento nos níveis de TBARS e carbonilas, sugerindo que a hiper-homocisteinemia crônica promoveu lipoperoxidação e dano a proteínas 1 e 12 h após a última administração de Hcy no tecido pulmonar. Esses resultados corroboram com dados prévios na literatura que demonstram que a administração de Hcy também promove lipoperoxidação em outros tecidos (Wyse et al., 2002; Matté et al., 2009a; Matté et al., 2009b). A produção de EROS e ERNS foi avaliada pelo ensaio de oxidação do DCF, o qual detecta radicais como peroxil, alcoxil, dióxido de nitrogênio, $OH^{\bullet}$ e $ONOO^{-}$ (Halliwell e Whiteman, 2004). Observamos que a hiper-homocisteinemia crônica provocou um aumento nos níveis de espécies reativas demonstrado pelo aumento nos níveis de DCF 1 e 12 h após a última injeção deste aminoácido. Sugerimos que a Hcy atua indiretamente através de sua auto-oxidação formando EROS e outros metabólitos já descritos anteriormente (Loscalzo, 1996; Jacobsen, 1998; Mansoor et al., 1995).

Outro parâmetro avaliado neste capítulo foi o TRAP que representa uma estimativa do potencial antioxidante não-enzimático (glutationa, ácido ascórbico, α-tacoferol, ácido úrico) (Evelson et al., 2001). Demonstramos que a administração crônica de Hcy promoveu uma diminuição significativa neste

parâmetro 12 h após a última injeção desse aminoácido, sugerindo que a Hcy promove uma redução nos níveis de antioxidantes não-enzimáticos no tecido pulmonar dos animais.

Considerando que o tratamento com Hcy promoveu uma alteração significativa no TRAP, resolvemos investigar o efeito da hiper-homocisteinemia crônica sobre as atividades das enzimas antixidantes enzimáticas (SOD, CAT e GPx). Essas enzimas oferecem um eficente sistema responsável pela remoção de EROS (Halliwell, 2001; Halliwell e Gutteridge, 2007). A SOD catalisa a dismutação do $O_2^{\bullet-}$, a CAT catalisa a redução do H_2O_2 e a GPx catalisa a dismutação tanto do H_2O_2 quanto de hidroperóxidos lipídicos (Grisham e McCord, 1986; Halliwell e Gutteridge, 2007). Os resultados demonstram que a administração de Hcy aumenta a atividade da SOD 1 h após a última injeção de Hcy, mas não verificamos alterações 12 h após a hiper-homocisteinemia crônica. Acreditamos que o aumento observado na atividade da SOD pode ter ocorrido em resposta do aumento dos níveis de $O_2^{\bullet-}$. Dados na literatura demonstram que a hiper-homocisteinemia promove um aumento dos níveis de $O_2^{\bullet-}$ através da ativação da enzima NADPH oxidase (Alvarez-Maqueda et al., 2004). É possível que esse aumento na atividade da SOD não foi suficiente para remover completamente o $O_2^{\bullet-}$ formado nesse modelo experimental de hiper-homocisteinemia crônica.

A Hcy também reduziu a atividade da CAT nos animais 1 e 12 h após hiper-homocisteinemia crônica. Por outro lado, verificamos uma redução na atividade da GPx somente nos animais eutanaziados 12 h após a última administração de Hcy. Já foi demonstrado pelo nosso grupo que a administração aguda de Hcy promoveu uma redução na atividade da CAT no

hipocampo de ratos e que o tratamento com vitamina E+C preveniu completamente esse efeito, indicando o envolvimento do estresse oxidativo nos efeitos da Hcy (Wyse et al., 2002). A diminuição nas atividades da CAT e GPx podem ser explicadas, pelo menos em parte, pelo fato de que as enzimas antioxidantes são inibidas pelas EROS (Blum e Fridorich, 1985; Fridorich, 1986; Vessey e Lee, 1993). Este desequilíbrio entre as enzimas antioxidantes provavelmente altera a eliminação de EROS (Halliwell, 2001; Halliwell e Gutteridge, 2007). Trabalhos demonstram que a Hcy estimula a produção de EROS e diminui a atividade da GPx, prejudicando a dismutação do H_2O_2 em células endoteliais de aorta (Upchurch et al., 1997b).

Observamos também que a Hcy reduziu os níveis de GSH e a atividade da enzima G6PD nos animais 1 e 12 h após a administração crônica de Hcy. A G6PD é uma enzima chave na regulação da via das pentoses por atuar como um redutor da coenzima nicotinamida adenina dinucleotídeo fosfato (NADP) à nicotinamida adenina dinucleotídeo fosfato reduzida (NADPH), sendo, praticamente, toda fonte geradora de NADPH. Ao manter a glutationa no estado reduzido (GSH), através da glutationa redutase, essa via desempenha importante papel na manutenção do estado redox celular (Kletzien et al., 1994). Assim, o resultado de diminuição da GPx pode estar relacionado com a diminuição nos níveis GSH e da atividade da G6PD encontradas neste estudo, uma vez que a atividade desta enzima depende da regeneração de GSH, que por sua vez depende de NADPH que é dependente de uma atividade normal de G6PD (Hashida et al., 2002). Dando continuidade a este estudo, investigamos os níveis de nitritos no tecido pulmonar dos ratos submetidos à administração crônica de Hcy. Não verificamos alterações significativas nos

níveis de nitritos no tecido pulmonar dos animais 1 e 12 h após a hiper-homocisteinemia crônica. O NO além de formar $ONOO^-$ (Beckman et al., 1990; Beckman e Koppenol, 1996) pode reagir com resíduos de tirosina para formar nitrotirosina (Eiserich et al., 1995). Neste contexto, Tyagi e cols. (2005) demonstraram que a Hcy aumenta significativamente os níveis de nitrotirosina sem alterar a atividade da NOS. Neste último trabalho da tese demonstramos que a Hcy crônica promove estresse oxidativo no tecido pulmonar dos animais, o que pode estar relacionado com o tromboembolismo pulmonar encontrado em alguns pacientes. Um resumo dos principais resultados observados no pulmão encontram-se no Anexo 3.

Demonstramos ao longo desse trabalho que a hiper-homocisteinemia induz uma alteração no sistema imune no cérebro e no sangue de ratos submetidos ao modelo experimental, além de alterar algumas vias de sinalização relacionadas com o processo inflamatório. Aliado ao processo inflamatório instalada nos animais também demonstramos um aumento no estresse oxidativo no sangue e no pulmão de ratos submetidos à hiper-homocisteinemia severa. Todos esses achados podem estar relacionados com as diferentes disfunções neurológicas e/ou periféricas encontradas nos pacientes homocistinúricos. Nossos resultados são de grande relevância e somam-se aos outros estudos do nosso grupo de pesquisa onde buscamos investigar mecanismos e possíveis agentes terapêuticos que visam previnir ou minimizar os efeitos encontrados nos pacientes homocistinúricos.

2. ANEXOS

ANEXO 1

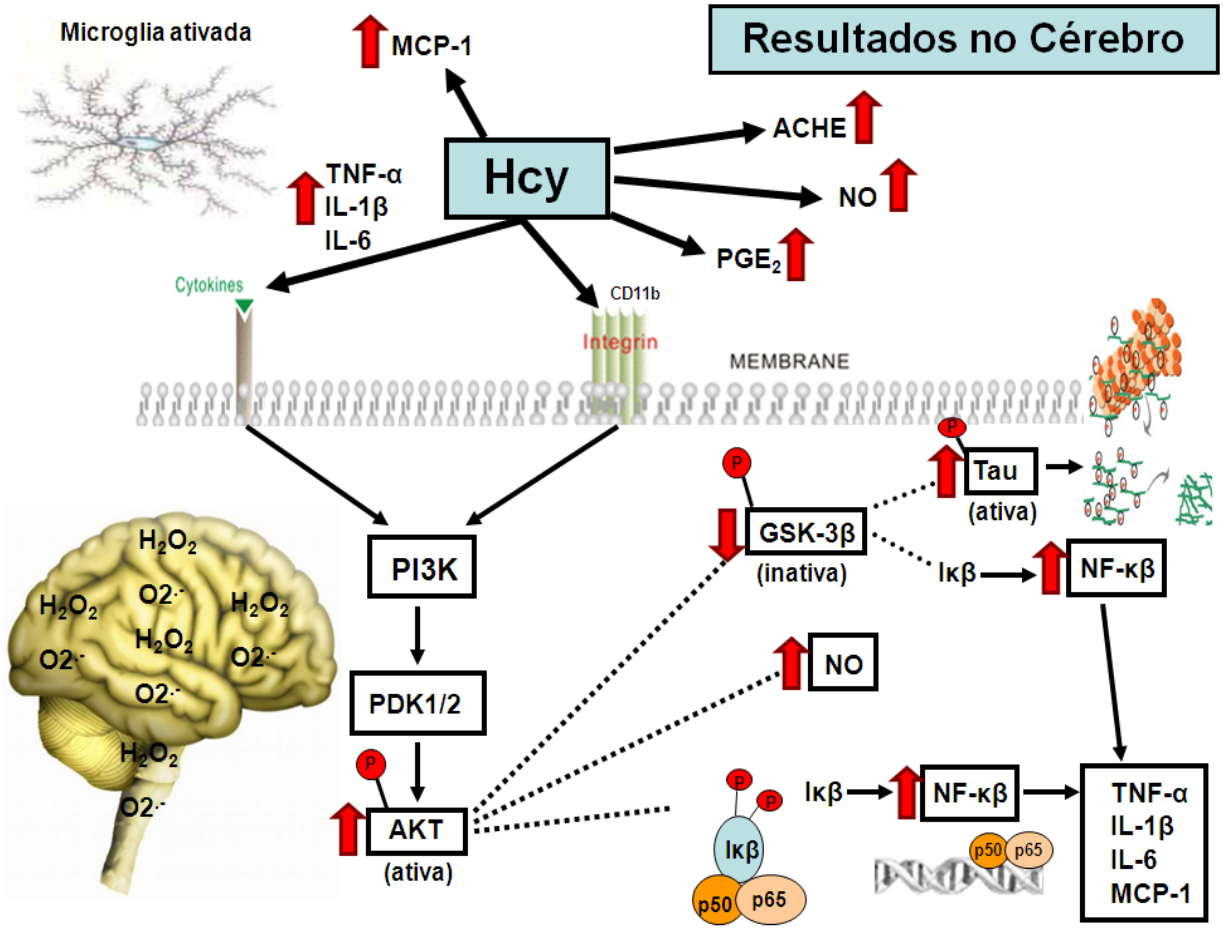

Figura 1: Resumo dos principais resultados encontrados no cérebro.

ANEXO 2

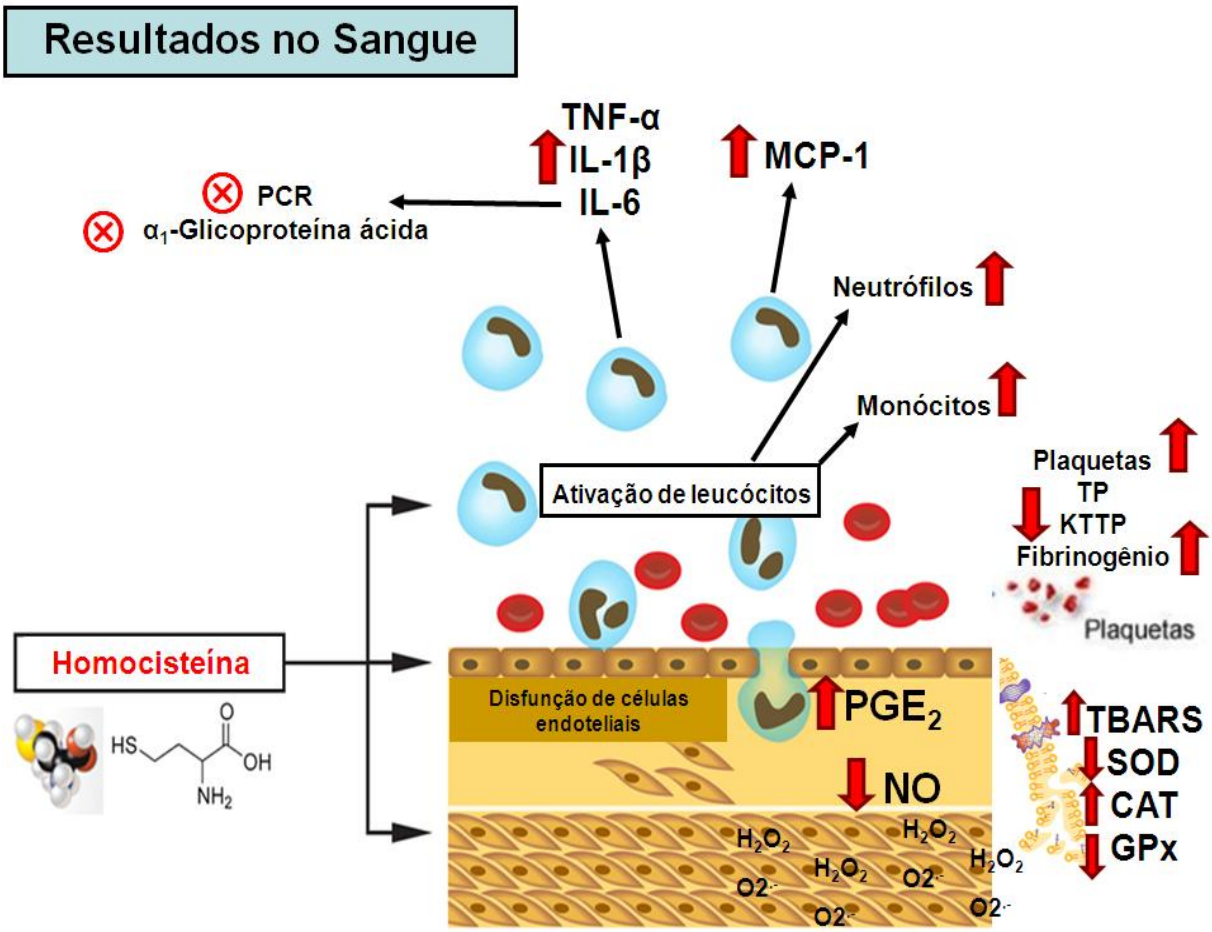

Figura 2: Principais resultados encontrados no sangue.

ANEXO 3

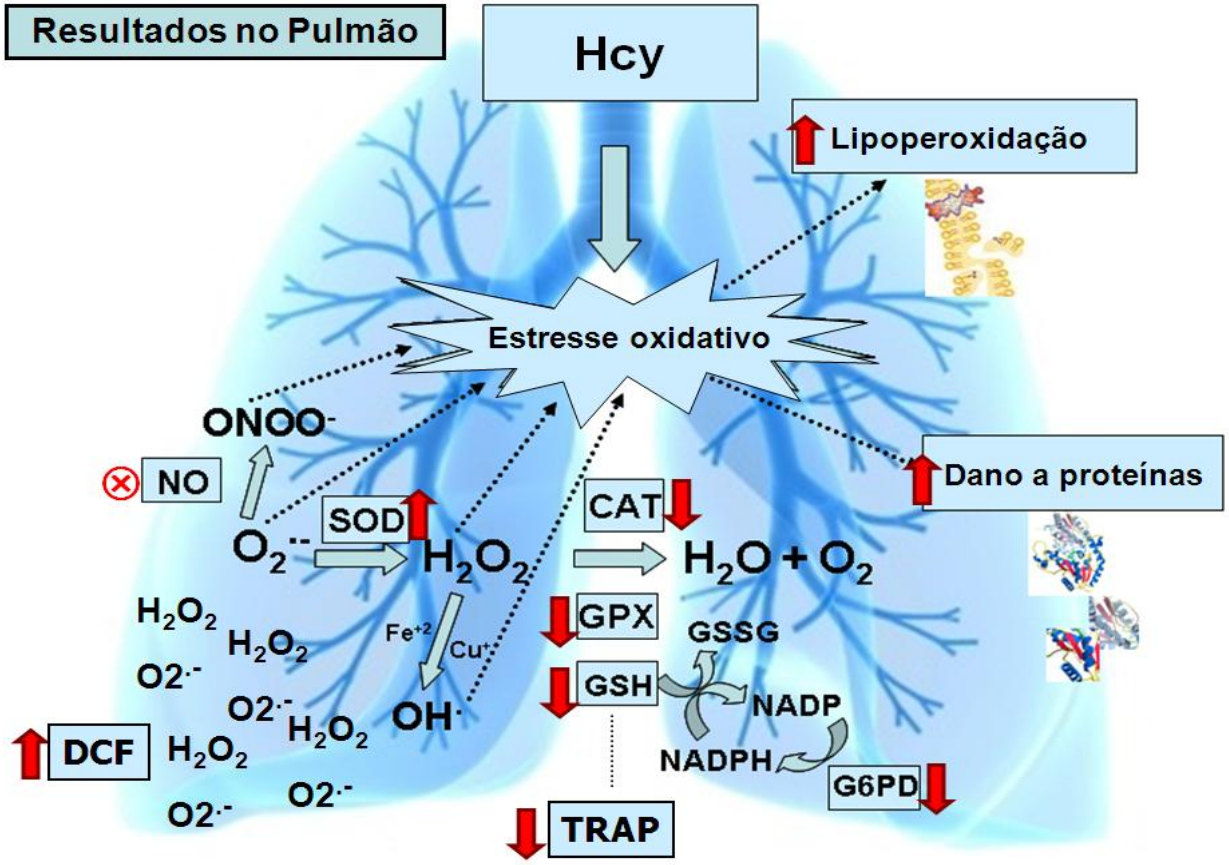

Figura 3: Principais resultados encontrados no pulmão.

3. CONCLUSÕES

Os resultados da presente tese permitem concluir que:

- ✓ A hiper-homocisteinemia aguda severa induz uma ativação do sistema imune pelo aumento nos níveis de citocinas (TNF-α, IL-1β, IL-6), quimiocina CCL_2 (MCP-1) e nitritos no hipocampo, córtex cerebral e/ou no soro dos ratos, além de promover um aumento relativo no número de neutrófilos e monócitos no sangue.
- ✓ A hiper-homocisteinemia crônica severa foi capaz de desencadear uma resposta inflamatória no hipocampo e no soro dos ratos, demonstrado pelo aumento nos níveis de citocinas (TNF-α, IL-1β, IL-6) e da quimiocina CCL_2 (MCP-1). Além disso, verificamos um aumento nos níveis de nitritos no hipocampo, e uma diminuição desse parâmetro no soro. Observou-se também um aumento nas frações citoplasmática e nuclear da subunidade p65 do NF-κB, um aumento nos níveis de PGE_2 e na atividade da acetilcolinesterase no hipocampo dos animais.
- ✓ A hiper-homocisteinemia aguda severa promove um estado de hipercoagulabilidade nos animais, verificado pelo aumento na contagem de plaquetas e de fibrinogênio no sangue e/ou plasma dos animais. Demonstramos também que a administração aguda desse aminoácido diminuiu o TP e KTTP e os níveis de nitritos no plasma.
- ✓ A hiper-homocisteinemia aguda severa induziu o estresse oxidativo no plasma de animais, determinado pelo aumento nos níveis de

TBARS e pelo desequilíbrio nas defesas antioxidantes enzimáticas (SOD, CAT e GPx) em eritrócitos de ratos.

- ✓ A hiper-homocisteinemia aguda severa aumentou a fosforilação da Akt, o imunoconteúdo das frações citoplasmática e nuclear da subunidade p65 do NF-κB e a fosforilação da proteína *Tau*, mas reduziu a fosforilação da GSK-3β no hipocampo de ratos.
- ✓ A hiper-homocisteinemia crônica severa não alterou a fosforilação da Akt e da GSK-3β no hipocampo de ratos.
- ✓ A hiper-homocisteinemia crônica severa induziu um estresse oxidativo no tecido pulmonar de ratos, evidenciado pelo aumento na lipoperoxidação e no dano a proteínas, bem como pela redução nas defesas antixidantes enzimáticas (SOD, CAT e GPx) e não-enzimáticas (TRAP), e pela diminuição dos níveis GSH e da atividade da G6PD.

3.1 CONCLUSÃO GERAL

Os resultados da presente tese mostram em conjunto, que a hiper-homocisteinemia aguda e crônica promoveu uma alteração no sistema imune em estruturas cerebrais e no sangue de ratos. Além disso, observamos alterações em importantes vias de sinalização intracelular relacionadas ao processo inflamatório e demonstramos que em nosso modelo experimental ocorre a indução de estresse oxidativo em eritrócitos e no tecido pulmonar. Esses achados podem estar relacionados com as disfunções cerebrais e cardiovasculares, bem como, com o tromboembolismo pulmonar que representam importantes características dessa patologia.

4. PERSPECTIVAS

- Avaliar o imunoconteúdo do CD11b, marcador de microglia ativada, no hipocampo de ratos submetidos aos modelos agudo e crônico de hiper-homocisteinemia severa;

- Determinar os níveis de NF-κB por Elisa no hipocampo de ratos submetidos aos modelos agudo e crônico de hiper-homocisteinemia severa;

- Avaliar o imunoconteúdo das enzimas iNOS e nNOS no hipocampo de ratos submetidos aos modelos agudo e crônico de hiper-homocisteinemia severa;

- Avaliar a migração de leucócitos através de microscopia intravital nos animais submetidos aos modelos agudo e crônico de hiper-homocisteinemia severa;

- Avaliar a atividade da enzima mieloperoxidase (MPO) no hipocampo de ratos submetidos aos modelos agudo e crônico de hiper-homocisteinemia severa;

- Investigar o efeito do tratamento com ácido fólico sobre as alterações nos parâmetros inflamatórios abordados nesta tese.

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6. LISTA DE FIGURAS