UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

METIONINA ALTERA PARÂMETROS BIOQUÍMICOS E COMPORTAMENTAIS

EM RATOS: ESTUDOS *IN VITRO* E *IN VIVO*

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ORIENTADORA

Profª. Drª. Angela Terezinha de Souza Wyse

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Doutor em Bioquímica

Porto Alegre, 2008

"Existe somente uma idade para a gente ser feliz, somente uma época na vida de cada pessoa em que é possível sonhar e fazer planos e ter energia bastante para realizá-los a despeito de todas as dificuldades e obstáculos...

... Tempo de entusiasmo e coragem em que todo desafio é mais um convite à luta que a gente enfrenta com toda disposição de tentar algo NOVO, de NOVO e de NOVO, e quantas vezes for preciso. Essa idade tão fugaz na vida da gente chama-se PRESENTE e tem a duração do instante que passa."

Mário Quintana

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SUMÁRIO

RESUMO

A hipermetioninemia ocorre em muitas doenças metabólicas, dentre elas, na deficiência da enzima metionina adenosiltransferase e na homocistinúria. Pacientes afetados por essas doenças podem apresentar alterações neurológicas e hepáticas; entretanto, os mecanismos responsáveis por essas manifestações não estão totalmente elucidados. No presente trabalho inicialmente avaliamos o efeito *in vitro* da metionina sobre a atividade da Na^+,K^+-ATPase e sobre alguns parâmetros de estresse oxidativo em hipocampo de ratos. Resultados mostraram que a pré-incubação de homogeneizados de hipocampo com metionina diminuiu a atividade da Na^+,K^+-ATPase e que antioxidantes (glutationa e trolox) preveniram esse efeito. Verificou-se também que a metionina diminuiu o potencial antioxidante total não enzimático (TRAP), aumentou a lipoperoxidação (medida pela quantidade de substâncias reativas ao ácido tiobarbitúrico – TBARS e pela quimiluminescência) e não alterou a atividade das enzimas antioxidantes catalase (CAT), glutationa peroxidase (GSH-Px) e superóxido dismutase (SOD). A seguir, desenvolvemos um modelo químico experimental de hipermetioninemia em ratos, a fim de estudar e melhor compreender os mecanismos fisiopatológicos dessa doença. Utilizando esse modelo, verificamos diversos parâmetros bioquímicos cerebrais, como a atividade da Na^+,K^+-ATPase e da acetilcolinesterase, alguns parâmetros de estresse oxidativo, o conteúdo lipídico total, bem como o aprendizado e a memória em ratos submetidos à tarefa do labirinto aquático de Morris. Também avaliamos parâmetros de estresse oxidativo em fígado de ratos hipermetioninêmicos. O modelo crônico de hipermetioninemia foi realizado do 6° ao 28° dia de vida e as doses de metionina administradas variaram de 1,34 a 2,68 µmol/g de peso corporal. Os ratos controles receberam solução salina no mesmo volume. As concentrações plasmáticas obtidas foram de 2 mmol/L, similares àquelas encontradas no plasma de pacientes hipermetioninêmicos. Os níveis cerebrais de metionina foram de aproximadamente 1 µmol/g de tecido. No tratamento agudo, ratos de 29 dias receberam uma injeção de metionina, na dose

de 2,68 µmol/g de peso corporal. Os resultados mostraram que a hipermetioninemia aguda e crônica aumentou o TBARS e reduziu a Na$^+$,K$^+$-ATPase em cérebro de ratos. A hipermetioninemia crônica reduziu o conteúdo total de gangliosídios, fosfolipídios e colesterol, e não alterou a CAT e o conteúdo tiólico total em cérebro de ratos. Também observamos que a administração crônica de metionina provocou um déficit na memória de trabalho e um aumento na atividade da acetilcolinesterase cerebral. Por outro lado, a administração aguda de metionina não alterou a atividade dessa enzima em cérebro de ratos. Por fim, demonstramos que a hipermetioninemia crônica aumentou a quimiluminescência, o conteúdo de carbonilas e a atividade da GSH-Px, e diminuiu o TRAP e a atividade da CAT em fígados de ratos. Em contraste, não houve alteração nos níveis de TBARS, TAR, conteúdo tiólico total e na atividade da SOD em fígado de ratos. Nossos achados, em conjunto, poderão auxiliar na compreensão das alterações neurológicas e hepáticas observadas em pacientes hipermetioninêmicos.

ABSTRACT

Hypermethioninemia is the biochemical hallmark of many metabolic disorders, such as methionine adenosyltransferase activity deficiency and homocystinuria. Affected patients can present neurological and hepatic alterations, whose underlying mechanisms are not yet fully established. In the present work, we evaluated the *in vitro* effect of methionine on Na^+,K^+-ATPase activity and some parameters of oxidative stress in hippocampus of rats. Results showed that incubation of homogenates of hippocampus with methionine diminished Na^+,K^+-ATPase activity and that simultaneous incubation with some antioxidants, such as glutathione and trolox prevented this effect. We also demonstrated that methionine decreased total radical antioxidant potential (TRAP), increased thiobarbituric acid reactive substances (TBARS) e chemiluminescence (both are markers of lipid peroxidation), but did not alter the activities of antioxidant enzymes catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in hippocampus of rats. Afterwards, considering that animal models are useful to better understand the physiopathology of human diseases, we developed a chemically induced experimental model of hypermethioninemia in rats. By using this model, we verified many biochemical parameters, as Na^+,K^+-ATPase and acetylcholinesterase activities, some oxidative stress parameters, total lipid content, as well as learning and memory in rats on Morris water maze task. We also determined the effect chronic hypermethioninemia on some parameters of oxidative stress in liver of rats. Chronic hypermethioninemia was performed from the 6th to the 28th day of life and methionine doses administered (1.34–2.68 μmol/g of body weight) were chosen in order to induce plasma levels similar to those described in hypermethioninemic patients (around to 2 mmol/L). By this treatment, we also produced high levels of methionine (approximately 1 μmol/g wet tissue) in brain of rats. Control rats received saline in the same volumes. In acute treatment, 29-day-old rats received one single injection of methionine (2.68 μmol/g of body weight). Results showed that acute and chronic administration of methionine

enhanced TBARS levels and decreased Na$^+$,K$^+$-ATPase activity in brain of rats. Chronic hypermethioninemia reduced total content of gangliosides, phospholipids and cholesterol, but did not change CAT activity and total thiol content brain of rats. We also observed that methionine-treated rats presented impaired performance on working memory task and an increase in brain acetylcholinesterase activity. On the other hand, acute administration did not alter this enzyme activity. Finally, we demonstrated that chronic hypermethioninemia increased chemiluminescence, protein carbonyl content and GSH-Px activity, and decreased TRAP and CAT activity in liver of rats. In contrast, TBARS, TAR, total thiol content and SOD activity were not affected by methionine administration in liver of rats. Altogether, our findings may be helpful in the understanding of the neurological and hepatic alterations observed in hypermethioninemic patients.

LISTA DE ABREVIATURAS

AChE – acetilcolinesterase

AdoHcy – *S*-adenosil-homocisteína

AdoMet – *S*-adenosilmetionina

AMPA – α-amino-3-hidroxi-5-metil-4-isoxazolpropionato

ATP – adenosina trifosfato

BuChE – butirilcolinesterase

CAT – catalase

DNA – ácido desoxirribonucléico

EIM – erros inatos do metabolismo

ERO – espécies reativas de oxigênio

GSH – glutationa reduzida

GSH-Px – glutationa peroxidase

HNE – 4-hidroxi-2-transnonenal

MAT – metionina adenosiltransferase

MDA – malondialdeído

mGluRs – receptores glutamatérgicos metabotrópicos

NMDA – *N*-metil-D-aspartato

RNA – ácido ribonucléico

SNC – sistema nervoso central

SOD – superóxido dismutase

TAR – reatividade antioxidante total

TBARS – substâncias reativas ao ácido tiobarbitúrico

TRAP – potencial antioxidante total

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I. INTRODUÇÃO

I.1 ERROS INATOS DO METABOLISMO

Os erros inatos do metabolismo (EIM) são doenças hereditárias, majoritariamente autossômicas recessivas, que se caracterizam pela síntese de proteínas alteradas, geralmente enzimas. Essas alterações resultam na diminuição total ou parcial da atividade enzimática, ocasionando o bloqueio de rotas metabólicas. Como conseqüência, pode ocorrer tanto o acúmulo de metabólitos tóxicos como a falta de produtos essenciais, ambos com doença subseqüente (Scriver et al., 2001).

A freqüência dos EIM é baixa, quando analisados individualmente; porém, em conjunto, estima-se que possam atingir um em cada mil recém-nascidos vivos. (Giugliani, 1988). Até o momento, já foram descritos mais de 500 EIM, a maioria envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas orgânicas (Scriver et al., 2001).

A classificação mais utilizada para os EIM é realizada de acordo com a área do metabolismo afetada e subdivide-se em EIM de: aminoácidos, ácidos orgânicos, glicídios, lipídios, glicosaminoglicanos, glicoproteínas, purinas e pirimidinas, enzimas eritrocitárias, metais, lipoproteínas, hormônios e proteínas plasmáticas (Scriver et al., 2001). Dentre os mais freqüentes, estão os EIM de aminoácidos, sendo exemplos a fenilcetonúria, a hiperprolinemia, a homocistinúria e a **hipermetioninemia**, que será alvo do nosso estudo.

I.2 METIONINA

A metionina é um aminoácido que provém da dieta ou da degradação de proteínas endógenas, sendo metabolizada principalmente no fígado. O primeiro passo no metabolismo desse composto é a formação de S-adenosilmetionina (AdoMet), em uma reação catalisada pela enzima metionina adenosiltransferase (MAT). A AdoMet, por sua vez, é convertida em S-adenosil-homocisteína (AdoHcy) ao doar grupos metila a diversos compostos como DNA, RNA, fosfolipídios e catecolaminas. Além de participar dessas reações de transmetilação, a AdoMet pode sofrer descarboxilação formando as poliaminas. O próximo passo é a hidrólise de AdoHcy em adenosina e homocisteína, através da enzima S-adenosil-homocisteína hidrolase (Finkelstein, 1990; Mudd et al., 2001).

A homocisteína formada pode ser metabolizada pelas vias de remetilação e transulfuração. Na via de remetilação, esse composto recebe um grupamento metila da betaína ou do 5-metiltetrahidrofolato formando metionina. Na transulfuração, a homocisteína, em uma reação catalisada pela enzima cistationina β-sintase, é convertida em cistationina. Essa, subseqüentemente, é hidrolisada a cisteína pela cistationina γ-liase. A cisteína pode participar da formação da glutationa reduzida (GSH), um importante antioxidante não enzimático, ou ser oxidada a taurina e sulfatos (Finkelsein, 1990; Mudd et al., 2001) (Figure 1). Sabe-se que sob condições nas quais há um excesso de metionina, além da transulfuração, existe outra via alternativa capaz de degradar esse aminoácido, a transaminação, na qual são formados metabólitos como o

metanotil e o sulfeto de hidrogênio (Benevenga & Steele, 1984; Mudd et al., 2001).
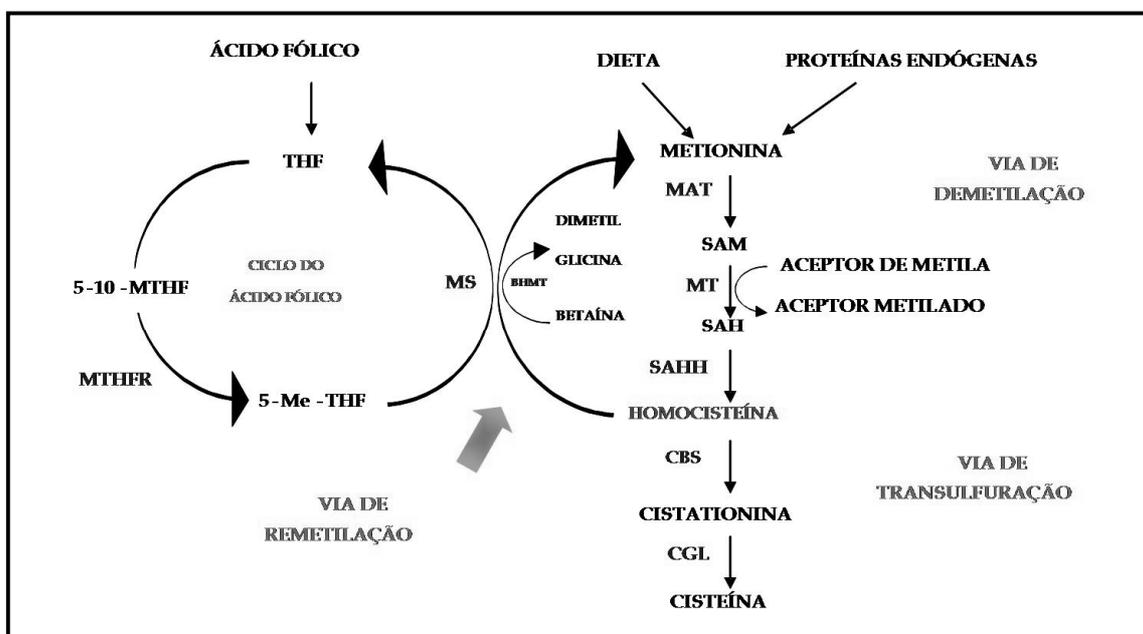
Figura 1. Metabolismo da metionina (Adaptado de Mudd et al., 2001).

MAT – metionina adenosiltransferase; **MT** – metiltransferase; **SAHH** – S-adenosil-homocisteína hidrolase; **CBS** – cistationina β-sintase; **CGL** – cistationina γ-liase; **MS** – metionina sintase; **BHMT** – betaína homocisteína metiltransferase; **MTHFR** – metilenotetrahidrofolato redutase; **SAM** – *S*-adenosilmetionina; **SAH** – *S*-adenosil-homocisteína; **THF** – tetrahidrofolato; **5,10-MTHF** – 5,10-metilenotetrahidrofolato; **5-MeTHF** – 5-metiltetrahidrofolato.

A metionina é essencial para o desenvolvimento e o crescimento normais de mamíferos e desempenha um papel importante na manutenção das metilações biológicas e na homeostase redox celular; portanto, seu metabolismo deve ser rigorosamente controlado (Prudova et al., 2005). Um ponto chave na regulação do ciclo da metionina é a concentração de AdoMet, que pode ativar ou inibir enzimas importantes, regulando assim, a velocidade da rota metabólica (Prudova et al., 2005; Finkelstein, 2006).

Existem trabalhos na literatura demonstrando que elevações nas concentrações de metionina podem causar efeitos deletérios em vários órgãos, como o cérebro e o fígado. Entretanto, os mecanismos responsáveis por esses efeitos são pouco conhecidos (Hardwick et al., 1970; Toborek et al., 1996; Mudd et al., 2001; Garlick et al., 2006).

A neurotoxicidade da metionina tem sido evidenciada através de estudos *in vitro* realizados em nosso grupo de pesquisa, demonstrando que esse aminoácido altera importantes parâmetros do metabolismo energético em cérebro de ratos, como a produção de CO_2, a liberação de lactato e a atividade da enzima Na^+,K^+-ATPase (Streck et al., 2002a, 2003). Além disso, estudos realizados em modelos animais demonstraram que a metionina apresenta efeito sinérgico sobre as convulsões causadas pela administração de homocisteína (Gaull et al., 1981; Labrune et al., 1990). Por outro lado, estudos clínicos evidenciaram níveis elevados de metionina no fluido cérebro-espinhal de pacientes psicóticos (Regland et al., 2004).

Alterações no metabolismo da metionina têm sido relacionadas à patogênese de diversas desordens hepáticas, como a cirrose e a esteatose (Tsukamato & Lu, 2001; Avila et al., 2005; Kharbanda, 2007). Neste contexto, Tsukamato e Lu (2001) demonstraram que a hipermetioninemia em pacientes cirróticos pode ser atribuída a uma redução de 50 a 60% na atividade da MAT. Por outro lado, Troen e colaboradores (2003) mostraram que altos níveis de metionina apresentam efeito aterogênico em camundongos com deficiência na apolipoproteína E.

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I.3 HIPERMETIONINEMIA

A hipermetioninemia pode ocorrer em várias desordens metabólicas, dentre elas, na deficiência da MAT, na homocistinúria (Mudd et al., 2000; 2001) e na deficiência da glicina *N*-metiltransferase (Augoustides-Savvopoulou et al., 2003). Nesse trabalho daremos ênfase à deficiência da MAT, pois é a causa genética mais comum de hipermetioninemia isolada (Mudd et al., 2001).

A deficiência da MAT é uma doença autossômica recessiva rara, caracterizada bioquimicamente pelo acúmulo tecidual do substrato metionina e pela falta do produto AdoMet. A concentração plasmática de metionina pode atingir até 2.500 μmol/L, sendo que os valores normais estão em torno de 30 μmol/L. Elevadas concentrações de metabólitos, como a metionina sulfóxido, o metanotiol e o sulfeto de hidrogênio, também podem ser observadas no plasma e na urina dos pacientes afetados por essa doença (Mudd et al., 2001).

Estudos genéticos demonstram que a deficiência da enzima ocorre devido a mutações no gene *MAT1A*, responsável por codificar as subunidades catalíticas das duas isozimas presentes em fígado adulto de mamíferos (MAT I e MAT III) (Mudd et al., 2000, 2001).

Embora alguns pacientes hipermetioninêmicos sejam assintomáticos, um número considerável apresenta alterações neurológicas como déficit cognitivo, edema e desmielinização cerebral, cuja fisiopatologia não está completamente estabelecida (Chamberlin et al., 1996; Mudd et al., 2000, 2001).

O diagnóstico é feito através da determinação da atividade da enzima hepática. Alterações nos níveis plasmáticos de AdoMet, na presença de alta concentração de metionina, podem auxiliar no diagnóstico. Apesar de permanecer controverso, o tratamento é baseado em uma dieta restrita em metionina, a fim de evitar os efeitos neurotóxicos do acúmulo desse aminoácido. Entretanto, essa restrição pode reduzir ainda mais os níveis de AdoMet, agravando os danos neurológicos presentes nos pacientes hipermetioninêmicos (Chien et al., 2005).

I.4 Na$^+$,K$^+$-ATPase

A Na$^+$,K$^+$-ATPase, também conhecida como bomba de Na$^+$-K$^+$, é uma proteína transmembrana responsável pela manutenção do gradiente iônico neuronal, através do transporte ativo de três íons Na$^+$ para o meio extracelular e de dois íons K$^+$ para o meio intracelular, com concomitante hidrólise de aproximadamente 50% do ATP produzido no cérebro (Ames, 2000; Erecinska et al., 2004).

O gradiente eletroquímico gerado pelo fluxo dos íons Na$^+$ e K$^+$ através da membrana celular é utilizado para a manutenção da excitabilidade neuronal, regulação do volume celular, do balanço osmótico e para o transporte de moléculas ligadas ao co-transporte de Na$^+$, como glicose, aminoácidos e neurotransmissores (Mobasheri et al., 2000; Kaplan, 2002; Jorgensen et al., 2003). Além de funcionar como uma bomba iônica, a Na$^+$,K$^+$-ATPase pode atuar como um receptor para a ouabaína nos eventos de transdução de sinal (Liu et al., 2003; Wang et al., 2004).

Estruturalmente, a Na^+,K^+-ATPase é uma enzima oligomérica composta de duas subunidades α, que contém os sítios de ligação para os íons Na^+ e K^+, ATP e glicosídios cardíacos, duas subunidades β, na forma de glicoproteínas, e uma subunidade γ, que está associada ao dímero $\alpha\beta$ (Mobasheri et al., 2000; Kaplan, 2002; Yu, 2003; Taguchi et al., 2007).

Durante o ciclo catalítico da Na^+,K^+-ATPase, a subunidade α é fosforilada e desfosforilada em um resíduo de ácido aspártico, estabilizando sua estrutura em duas conformações, E1 e E2. Na conformação E1, ocorre a ligação de três íons Na^+ na face intracelular, favorecendo a transferência de um grupo fosfato do ATP para o sítio ativo da enzima. Essa fosforilação provoca uma mudança conformacional na Na^+,K^+-ATPase, com conseqüente liberação dos íons Na^+ para o meio extracelular. Na conformação E2, a enzima apresenta alta afinidade por íons K^+, ocorrendo assim, a ligação de dois íons na face extracelular, o que provoca a desfosforilação da enzima e, conseqüentemente, a liberação desses íons para o meio intracelular. A enzima desprovida do grupamento fosfato não é estável na forma E2, voltando à forma E1, que tem alta afinidade por Na^+ (Mobasheri et al., 2000; Kaplan, 2002; Jorgensen et al., 2003) (Figura 2).
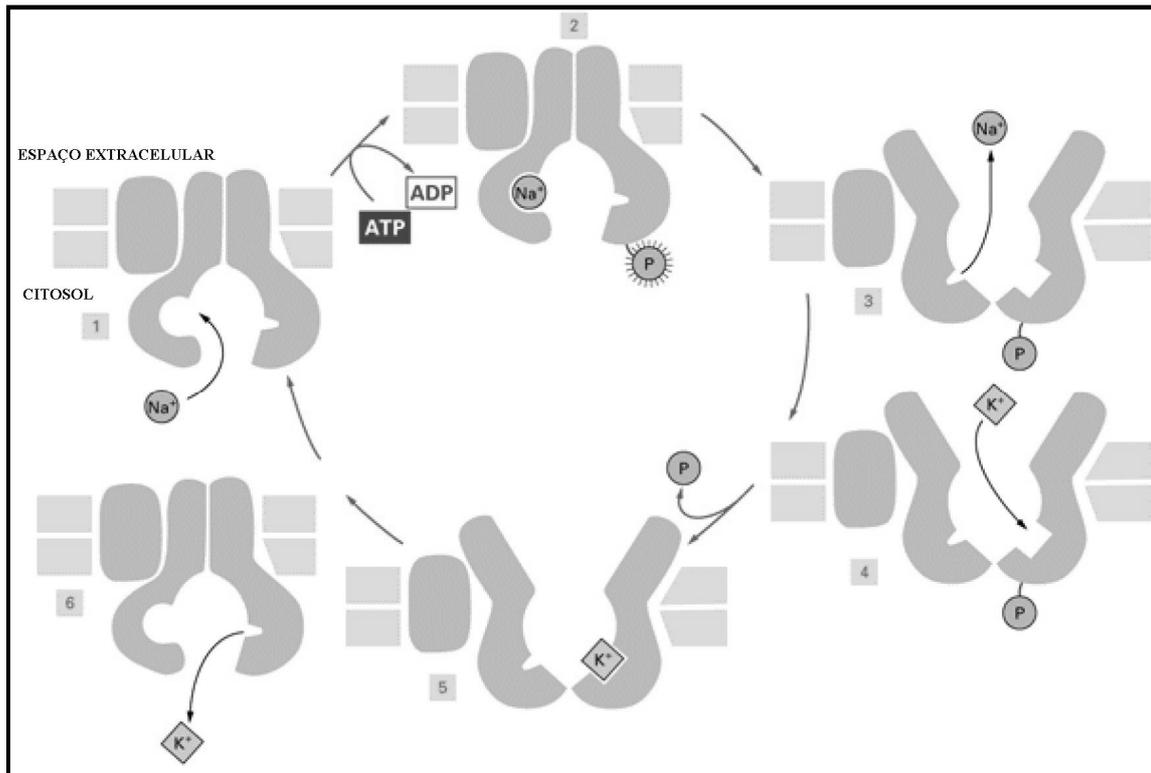
Figura 2. Ciclo catalítico da Na$^+$,K$^+$-ATPase (Adaptado de Alberts et al., 2004).

Considerando que a Na$^+$,K$^+$-ATPase é importante para funções celulares e sinápticas, a inibição da atividade dessa enzima pode ocasionar prejuízo no funcionamento normal do sistema nervoso central (SNC). Neste contexto, estudos mostraram que a ouabaína, um potente e seletivo inibidor da Na$^+$,K$^+$-ATPase, apresenta ação neurotóxica, podendo ocasionar morte neuronal (Lees & Leong, 1995; Yu, 2003). Adicionalmente, tem sido demonstrada uma associação entre a diminuição da atividade dessa enzima e a fisiopatologia de diversas doenças que afetam o SNC, tais como a isquemia cerebral (Wyse et al., 2000), as doenças neurodegenerativas (Yu, 2003; Vignini et al., 2007) e as desordens depressivas (Goldstein et al., 2006).

Estudos prévios realizados em nosso grupo de pesquisa mostraram que alguns aminoácidos e/ou seus metabólitos acumulados em EIM, tais como a fenilalanina (Wyse et al., 1999), a prolina (Franzon et al., 2003), a arginina (Bavaresco et al., 2003) e a homocisteína (Streck et al., 2002a; Matté et al., 2004) inibem a atividade da Na^+,K^+-ATPase em cérebro de ratos.

Tem sido demonstrado que a Na^+,K^+-ATPase é altamente vulnerável ao insulto oxidativo, sendo inibida por espécies reativas de oxigênio (ERO) (Kurella et al., 1999; Wang et al., 2003), possivelmente através da oxidação de grupamentos tiólicos essenciais para a atividade da enzima (Dobrota et al., 1999; Kurella et al., 1999). Essa enzima também pode ser inibida por produtos da peroxidação lipídica, como malondialdeído (MDA) e 4-hidroxi-2-transnonenal (HNE) e por alterações na fluidez da membrana plasmática (Lehotsky et al., 1999; Rauchová et al., 1999; Chakraborty et al., 2003).

I.5 RADICAIS LIVRES E ESTRESSE OXIDATIVO

Um radical livre é definido como qualquer espécie química capaz de existir de forma independente e que contenha um ou mais elétrons desemparelhados. Os radicais são altamente reativos e formados pela perda ou pelo ganho de um elétron por um não radical, bem como pelo processo de fissão homolítica, o qual corresponde à quebra de uma ligação covalente (Halliwell & Gutteridge, 2007).

As ERO – termo utilizado para designar radicais (ânion superóxido e radical hidroxila) e alguns não radicais derivados do oxigênio (peróxido de hidrogênio e oxigênio singleto) – são formadas principalmente durante a respiração celular,

pela redução incompleta (2-5%) do oxigênio molecular (Salvador & Henriques, 2004; Halliwell & Gutteridge, 2007). Fisiologicamente, essas espécies participam de funções importantes, como a fagocitose, a sinalização celular, a regulação de proteínas e a plasticidade sináptica (Ward & Peters, 1995; Serrano & Klann, 2004; Halliwell & Gutteridge, 2007). Entretanto, quando em excesso, as ERO podem oxidar diversas biomoléculas, como os lipídios, as proteínas e o DNA. O dano oxidativo aos lipídios, ou lipoperoxidação, ocorre quando as ERO reagem com lipídios insaturados presentes nas membranas celulares, ocasionando modificações na permeabilidade e na fluidez da membrana, além de causar dano a proteínas transmembrana, como enzimas, receptores e canais iônicos (Halliwell & Gutteridge, 2007) (Figura 3).
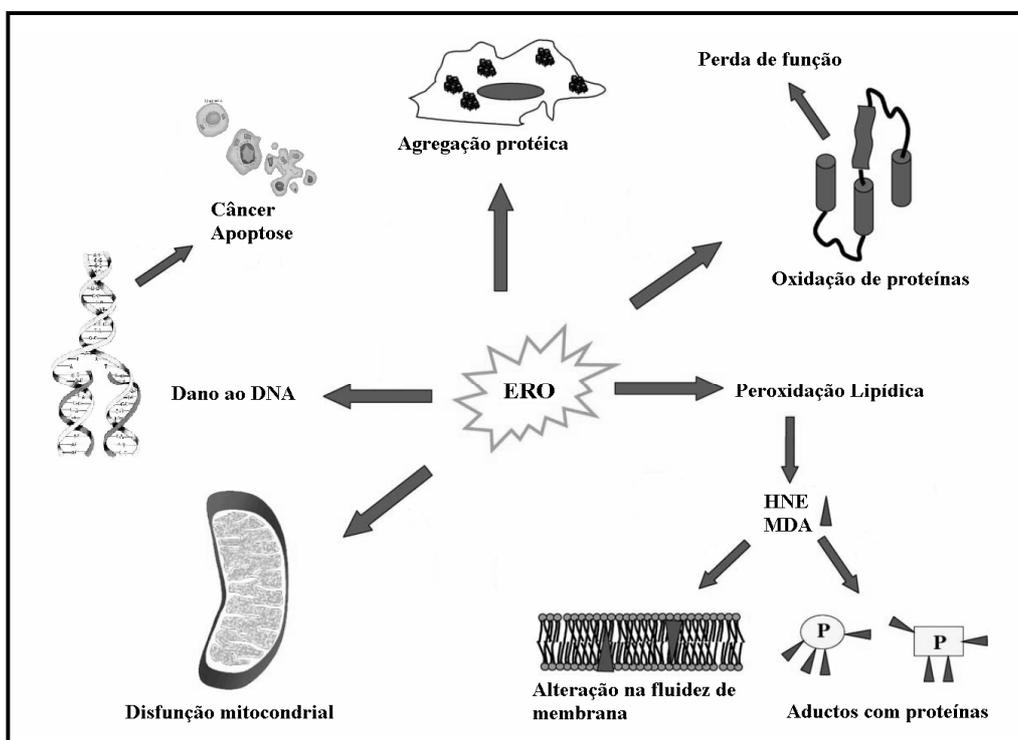
Figura 3. Dano oxidativo às biomoléculas.

A fim de evitar os efeitos danosos das espécies reativas, o nosso organismo dispõe de mecanismos eficientes para a detoxificação desses agentes oxidantes, conhecidos como defesas antioxidantes. Essas podem ser divididas em enzimáticas e não enzimáticas. As principais enzimas antioxidantes são a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GSH-Px). As defesas antioxidantes não enzimáticas incluem principalmente a GSH, o ácido ascórbico (vitamina C), o α-tocoferol (vitamina E), os polifenóis, a melatonina, a bilirrubina, o urato, o ácido lipóico e os estrógenos (Salvador & Henriques, 2004; Halliwell & Gutteridge, 2007).

Em condições fisiológicas, há um balanço entre a produção de espécies reativas e os sistemas de defesa antioxidante; porém, em certas condições patológicas pode haver aumento da produção de oxidantes e/ou diminuição dos níveis de antioxidantes, favorecendo a ocorrência do estresse oxidativo (Halliwell & Gutteridge, 2007).

O cérebro é altamente suscetível ao dano oxidativo, uma vez que apresenta elevado consumo de oxigênio, presença de neurotransmissores auto-oxidáveis, membrana neuronal rica em ácidos graxos poliinsaturados, alto nível de ferro e modesta defesa antioxidante (Halliwell, 2006). Neste sentido, existem diversas evidências sugerindo o envolvimento do estresse oxidativo na patogênese do dano neurológico presente em várias doenças neurodegenerativas, tais como as doenças de Alzheimer e Parkinson, e a esclerose amiotrófica lateral (Mancuso et al., 2006; Halliwell, 2006). Além disso, trabalhos realizados em nosso laboratório demonstraram que em modelos animais de EIM, como a hiperprolinemia (Delwing

et al., 2003), a hiperargininemia (Wyse et al., 2001) e a homocistinúria (Matté et al., 2004), há indução de estresse oxidativo cerebral.

Sabe-se que o fígado possui alta resistência ao dano causado pelos radicais livres, pois dispõe de elevados níveis de antioxidantes e pode se adaptar facilmente às alterações metabólicas (Genet et al., 2002). No entanto, o estresse oxidativo tem sido reconhecido como um fator fundamental nas diversas mudanças fisiopatológicas observadas em doenças hepáticas, como a hepatite aguda, a cirrose hepática e o carcinoma hepatocelular (Loguercio & Federico, 2003; Kim et al., 2004; Tanikawa & Torimura, 2006). Neste contexto, Yalçinkaya e colaboradores (2007) demonstraram que uma dieta suplementada com metionina aumenta o dano oxidativo e a hepatoxicidade em ratos cronicamente tratados com etanol. Além disso, a hipermetioninemia altera o status pró-oxidante/antioxidante em fígado de ratos (Toborek et al., 1996; Mori & Hirayama, 2000).

I.6 LIPÍDIOS DE MEMBRANA

As membranas celulares são compostas por três classes principais de lipídios: esfingolipídios, fosfolipídios e colesterol, os quais desempenham uma série de funções fisiológicas importantes (Agranoff & Hajra, 1994; Devlin, 2003).

Os gangliosídios são glicoesfingolipídios complexos, com um ou mais resíduos de ácido siálico na molécula, altamente concentrados nas membranas celulares neurais (Tettamanti & Riboni, 1994; Devlin, 2003; Sonnino et al., 2007). Os principais gangliosídios presentes no cérebro são GM1, GD1a, GD1b e GT1b, os quais são sintetizados a partir de um precursor comum derivado da

lactosilceramida (Kolter et al., 2002). Esses lipídios apresentam uma variedade de funções importantes nos neurônios, como a participação na proliferação e diferenciação neuronal, na adesão celular, na mielinização e na transmissão sináptica (Nagai, 1995; Kolter et al., 2002; Mocchetti et al., 2005). Adicionalmente, She e colaboradores (2005) demonstraram que os gangliosídios afetam a plasticidade sináptica em hipocampo, sendo efetivos para atenuar déficits cognitivos em ratos.

Os fosfolipídios compreendem um grupo heterogêneo de compostos que geralmente apresentam as seguintes regiões: grupo cabeça polar unido ao glicerol por uma ponte fosfodiéster e cadeias hidrocarbonadas longas. Esses lipídios constituem o esqueleto das membranas neurais e conferem a essas, fluidez e permeabilidade iônica; além disso, são requeridos para a função de proteínas transmembrana, como receptores e canais iônicos. Os fosfolipídios mais abundantes são a fosfatidilcolina, a fosfatidiletanolamina e a fosfatidilserina (Devlin, 2003; Farooqui et al., 2004). A esfingomielina, também considerada um fosfolipídio por apresentar a fosfocolina como grupo cabeça polar, está presente em altas concentrações na mielina (Devlin, 2003).

O colesterol, quimicamente derivado do ciclopentanoperidrofenantreno, também é um constituinte integral das membranas plasmáticas, localizando-se especialmente no tecido neural (Ohvo-Rekilä et al., 2002; Devlin, 2003). Apresenta diversas funções como a modulação de propriedades físico-químicas das membranas, a formação da mielina, a sinaptogênese e a liberação de neurotransmissores (Mauch et al., 2001; Valenza & Cattaneo, 2006). O colesterol - juntamente com os glicoesfingolipídios - está concentrado em microdomínios de

membrana resistentes à solubilização com detergentes, denominados *lipid rafts*, os quais parecem ser plataformas para os eventos de transdução de sinal (Paratcha & Ibanez, 2002).

Sabe-se que alterações na composição lipídica podem acarretar mudanças nas propriedades físicas das membranas e conseqüentemente, ocasionar disfunção neuronal (Tettamanti & Riboni, 1994; Farooqui et al., 2004; Swapna et al., 2006; Valenza & Cattaneo, 2006). Neste contexto, variações no conteúdo e na composição dos gangliosídios têm sido observadas em danos cerebrais como a hipóxia (Yin et al., 2006), a isquemia (Kwak et al., 2005) e a doença de Alzheimer (Barrier et al., 2007). Adicionalmente, há estudos demonstrando que o conteúdo cerebral de colesterol e de fosfolipídio diminui com o avanço da idade (Svennerholm et al., 1994) e em pacientes com desordens neurodegenerativas (Svennerholm & Gottfries, 1994; Farooqui et al., 2004).

I.7 ACETILCOLINESTERASE

A acetilcolina é um neurotransmissor clássico que apresenta papel importante no controle de diversos processos fisiológicos no SNC e no periférico. É sintetizada pela enzima colina acetiltransferase a partir de acetato e colina, armazenada em vesículas no neurônio pré-sináptico e tem sua ação finalizada pelas colinesterases presentes nas sinapses (Prado et al., 2002).

As colinesterases, acetilcolinesterase (AChE) e butirilcolinesterase (BuChE), são constituintes ubíquos do sistema colinérgico e diferem basicamente quanto à distribuição tecidual, propriedade cinética, especificidade por substratos

e por inibidores seletivos (Massoulié et al., 1993). A AChE está presente em maior concentração no SNC, na junção neuromuscular e na membrana de eritrócitos, hidrolisando preferencialmente a acetilcolina (Massoulié et al., 1993; Dave et al., 2000).

Quanto à estrutura, a AChE pertence à família de proteínas α/β, pois contém uma folha β central rodeada por hélices α (Soreq & Seidman, 2001; Darvesh et al., 2003). O sítio ativo é formado por um sítio esterásico, que contém uma tríade catalítica composta pelos aminoácidos serina, histidina e glutamato e por um sítio de ligação da colina ou aniônico (Soreq & Seidman, 2001; Lane et al., 2006; Zimmerman & Soreq, 2006). Existem duas formas moleculares da AChE: assimétrica e globular. A forma globular é composta por monômeros, dímeros ou tetrâmeros da subunidade catalítica, localizando-se no tecido nervoso, enquanto a assimétrica consiste de um, dois ou três tetrâmeros catalíticos ligados covalentemente a uma subunidade não catalítica e está mais concentrada na junção neuromuscular (Aldunate et al., 2004; Lane et al., 2006).

Além de seu papel clássico na transmissão colinérgica, há dados na literatura demonstrando que a AChE contribui para a transmissão sináptica dopaminérgica e glutamatérgica (Zimmerman & Soreq, 2006). Também está envolvida na adesão celular (Soreq & Seidman, 2001), no crescimento de neuritos (Day & Greenfield, 2002), na sinaptogênese (Soreq & Seidman, 2001), no fluxo sangüíneo cerebral, na modulação da glia, na cascata amilóide e na fosforilação da proteína *tau* (Ballard et al., 2005; Lane et al., 2006).

Muitos estudos têm evidenciado a relação entre a doença de Alzheimer e o sistema colinérgico central, uma vez que a diminuição da neurotransmissão colinérgica contribui, pelo menos em parte, para o déficit cognitivo e os distúrbios comportamentais observados em pacientes com essa desordem (Ballard et al., 2005; Fodale et al., 2006). Neste contexto, Pillay e colaboradores (2003) mostraram que a acetilcolina apresenta um papel neuroprotetor, sugerindo que a diminuição desse neurotransmissor no SNC poderia acelerar a neurodegeneração observada em pacientes com a doença de Alzheimer. Adicionalmente, tem sido demonstrado um envolvimento do estresse oxidativo no aumento da atividade da AChE, com conseqüente redução da acetilcolina, em cultura de células de pacientes com Alzheimer (Melo et al., 2003).

Convém ressaltar que a AChE tem sido investigada como um importante alvo no tratamento de várias doenças neurodegenerativas. Neste contexto, os inibidores reversíveis dessa enzima, utilizados na clínica, melhoram os sintomas cognitivos, comportamentais e funcionais relacionados às demências hipocolinérgicas, como a doença de Alzheimer (Giacobini, 2003; Lane et al., 2006).

I.8 MEMÓRIA

O aprendizado e a memória são funções essenciais do SNC; entretanto, apesar de intimamente relacionadas, compreendem processos distintos. O aprendizado pode ser definido como uma alteração relativamente permanente no comportamento que ocorre em conseqüência da prática ou da experiência, já a

memória consiste na capacidade de armazenar e evocar informações (Lent, 2002; Squire & Kandel, 2003).

Para que ocorra a formação da memória deve haver inicialmente a aquisição da informação, que consiste na entrada de um evento qualquer nos sistemas neurais ligados à memória. Porém, dependendo do tipo de informação, ela pode ser esquecida imediatamente, memorizada por um curto período, ou retida por períodos prolongados, o que caracteriza o processo de consolidação. Por fim, ocorre a evocação, processo pelo qual a informação armazenada pode ser requisitada para uso na cognição, emoção e/ou expressão de um comportamento (Izquierdo, 2002).

As memórias podem ser classificadas quanto ao tempo de retenção da informação armazenada em: memória imediata ou sensorial, com duração de apenas alguns segundos; memória de curta duração, podendo durar minutos ou poucas horas; e memória de longa duração, que pode durar horas, dias ou anos, garantindo o registro do passado autobiográfico e dos conhecimentos do indivíduo (Izquierdo & McGaugh, 2000; Squire & Kandel, 2003).

Embora os mecanismos envolvidos nos processos de formação da memória não estejam completamente estabelecidos, há diversos trabalhos na literatura sugerindo a participação de uma série de alterações bioquímicas em diferentes áreas do SNC. Os eventos bioquímicos incluem, inicialmente, a ativação de receptores glutamatérgicos dos tipos NMDA (*N*-metil-D-aspartato), AMPA (α-amino-3-hidroxi-5-metil-4-isoxazolpropionato) e metabotrópicos (mGluRs), seguida de mudanças em segundos mensageiros com ativação de proteínas cinases

(Izquierdo & Medina, 1997; Izquierdo, 2002). Além disso, tem sido proposto o envolvimento da Na^+,K^+-ATPase (Sato et al., 2004; Wyse et al., 2004) e do estresse oxidativo (Abidin et al., 2004; Silva et al., 2004) na modulação dos processos cognitivos.

Atualmente, existem várias técnicas capazes de avaliar a memória/aprendizagem em animais, sendo uma delas, a tarefa do labirinto aquático de Morris. Esse teste é utilizado para investigar o aprendizado e a memória espacial (D'Hooge & De Deyn, 2001). Estudos mostram que o aprendizado espacial em geral depende da ação coordenada dos sistemas de neurotransmissão e de diferentes regiões cerebrais incluindo principalmente o hipocampo. Corroborando com esses dados, investigadores demonstraram que lesões nessa estrutura, no córtex cerebral e no estriado, parecem prejudicar o desempenho dos animais na tarefa do labirinto aquático de Morris (D'Hooge & De Deyn, 2001).

I.9 OBJETIVOS

Objetivo Geral

Com a finalidade de melhor compreender os mecanismos envolvidos nas alterações neurológicas presentes na hipermetioninemia, o objetivo geral desse trabalho foi investigar o efeito da metionina sobre alguns parâmetros bioquímicos (atividade da Na^+,K^+-ATPase e da AChE, parâmetros de estresse oxidativo, conteúdo e composição de lipídios de membrana) em cérebro de ratos, bem como avaliar possíveis alterações comportamentais em ratos submetidos à hipermetioninemia. Considerando que a hipermetioninemia pode causar dano hepático, alguns parâmetros de estresse oxidativo também foram avaliados em fígado de ratos hipermetioninêmicos.

Esse trabalho será dividido em seis capítulos como segue:

Capítulo I

Objetivos Específicos

1. Investigar o efeito da pré-incubação de homogeneizados de hipocampo de ratos na presença de metionina sobre a atividade da Na^+,K^+-ATPase em membrana plasmática sináptica.

2. Avaliar o papel dos antioxidantes GSH e trolox sobre a inibição da Na^+,K^+-ATPase causada pela metionina.

3. Investigar o efeito *in vitro* de diferentes concentrações de metionina sobre alguns parâmetros de estresse oxidativo, denominados quimiluminescência, substâncias reativas ao ácido tiobarbitúrico (TBARS), potencial antioxidante

total (TRAP), bem como sobre a atividade das enzimas antioxidantes CAT, GSH-Px e SOD em hipocampo de ratos.

Capítulo II

Objetivos Específicos

1. Desenvolver um modelo químico experimental de hipermetioninemia em ratos jovens, a fim de mimetizar, pelo menos em parte, a doença que ocorre em humanos.

Capítulo III

Objetivos Específicos

1. Avaliar o efeito da administração crônica de metionina sobre alguns parâmetros de estresse oxidativo, tais como TBARS, atividade da enzima CAT e conteúdo tiólico total, bem como sobre a atividade da Na^+,K^+ - ATPase em hipocampo de ratos.

2. Verificar o efeito da hipermetioninemia aguda sobre a atividade da Na^+,K^+ - ATPase e sobre os níveis de TBARS em hipocampo de ratos.

Capítulo IV

Objetivos Específicos

1. Avaliar o efeito da administração crônica de metionina sobre o conteúdo e a distribuição de gangliosídios e fosfolipídios, bem como sobre a concentração de colesterol em córtex cerebral de ratos.

2. Verificar o efeito da hipermetioninemia crônica sobre a atividade da Na^+,K^+-ATPase e sobre os níveis de TBARS em córtex cerebral de ratos.

Capítulo V

Objetivos Específicos

1. Avaliar o aprendizado e a memória espacial na tarefa do labirinto aquático de Morris em ratos adultos submetidos ao modelo químico experimental de hipermetioninemia.

2. Verificar o efeito da administração aguda e crônica de metionina sobre a atividade da AChE em córtex cerebral de ratos.

Capítulo VI

Objetivos Específicos

1. Verificar o efeito da administração crônica de metionina sobre parâmetros de estresse oxidativo, denominados quimiluminescência, TBARS, TRAP, reatividade antioxidante total (TAR), conteúdo tiólico total e de carbonilas, bem como sobre a atividade das enzimas antioxidantes CAT, GSH-Px e SOD em fígado de ratos.

OBS: Todos os capítulos serão apresentados na forma de artigos científicos.

II. ARTIGOS CIENTÍFICOS

CAPÍTULO I – ARTIGO 1

Methionine alters Na$^+$,K$^+$-ATPase activity, lipid peroxidation and nonenzymatic antioxidant defenses in rat hippocampus

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Methionine alters Na$^+$,K$^+$-ATPase activity, lipid peroxidation and nonenzymatic antioxidant defenses in rat hippocampus

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Abstract

In the present study we investigated the effect of methionine exposure of hippocampus homogenates on Na$^+$,K$^+$-ATPase activity from synaptic plasma membrane of rats. Results showed that methionine significantly decreased this enzyme activity. We also evaluated the effect of incubating glutathione (GSH) and trolox (α-tocopherol) alone or combined with methionine on Na$^+$,K$^+$-ATPase activity. The tested antioxidants per se did not alter the enzymatic activity, but prevented the inhibitory action of methionine on Na$^+$,K$^+$-ATPase activity, indicating that Met inhibitory effect was probably mediated by free radical formation. Besides, we tested the in vitro effect of methionine on some parameters of oxidative stress, namely chemiluminescence, thiobarbituric acid reactive substances (TBARS), total radical-trapping antioxidant potential (TRAP), as well as on the antioxidant enzyme activities catalase, glutathione peroxidase and superoxide dismutase in rat hippocampus. We observed that methionine significantly increased chemiluminescence and TBARS, decreased TRAP, but did not change the activity of the antioxidant enzymes. These findings suggest that reduction of Na$^+$,K$^+$-ATPase activity and induction of oxidative stress may be involved in the brain damage observed in human hypermethioninemia.
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Keywords: Methionine; Na$^+$,K$^+$-ATPase; Oxidative stress; Antioxidants; Hippocampus; Rat

1. Introduction

Hypermethioninemia is the biochemical hallmark of methionine adenosyltransferase (MAT, EC 2.5.1.6) activity deficiency, as well as of homocystinuria. Affected patients with these disorders present neurological manifestations, including cognitive deficit, mental retardation, cerebral edema and demyelination, whose underlying mechanisms are not yet fully established (Chamberlin et al., 1996; Mudd et al., 2000; Mudd et al., 2001).

Na$^+$,K$^+$-ATPase (EC 3.6.1.37) is a crucial enzyme responsible for maintaining the ionic gradient necessary for neuronal excitability. It is present at high concentrations in brain cellular membranes, consuming about 40–50% of the ATP generated in this tissue (Erecinska and Silver, 1994). It has been demonstrated that this enzyme is susceptible to free radical attack (Lees, 1993). Besides, there are some reports showing that Na$^+$,K$^+$-ATPase activity is decreased in cerebral ischemia (Wyse et al., 2000), in epilepsy (Grisar et al., 1992) and in various chronic neurodegenerative disorders (Lees, 1993).

On the other hand, there is considerable evidence showing that oxidative stress is an important event occurring in various common acute and chronic neurodegenerative pathologies, such as seizures, cerebral ischemia, demyelination, dementia and Alzheimer's disease (Halliwell and Gutteridge, 1985; Reznick and Packer, 1993; Karelson et al., 2001; Méndez-Álvarez et al., 2001). This is understandable since the central nervous system is potentially sensitive to oxidative damage due to its great oxygen consumption, high lipid content and poor antioxidant defenses (Halliwell, 1996).

We have recently shown that methionine (Met) added to the enzymatic assay, at concentrations usually found in homocystinuria, inhibits Na$^+$,K$^+$-ATPase in purified

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synaptic plasma membrane preparations from hippocampus of rats, suggesting a direct effect of this amino acid on this enzyme activity (Streck et al., 2002).

In the present study we investigated the effect of exposing hippocampus homogenates to Met on Na^+,K^+-ATPase activity from rat synaptic plasma membranes. We also evaluated the role of the antioxidants glutathione (GSH) and trolox (α-tocopherol) on the inhibition of this enzyme activity caused by Met. We finally assessed whether Met could induce oxidative stress by measuring some parameters of oxidative stress, namely chemiluminescence, thiobarbituric acid reactive substances (TBARS), total radical-trapping antioxidant potential (TRAP), and the activities of the antioxidant enzymes catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD).

2. Experimental procedure

2.1. Subjects and reagents

Thirty-seven Wistar rats with 29-day-old were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Science of Health, Federal University of 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 ($22 \pm 1\ ^{\circ}C$) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. The chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Tissue and homogenate preparation

Animals were killed by decapitation without anaesthesia, the brain was quickly removed and the hippocampus was dissected and homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4. The homogenates were separately incubated at 37 °C for 1 h with Met (0.02–5.0 mM), 1.0 mM GSH, 3.0 mM trolox, and with 5.0 mM Met combined with 1.0 mM GSH or 3.0 mM trolox. All substances were dissolved in Tris–HCl buffer, pH 7.4. After incubation, synaptic plasma membranes were prepared as described below and the activity of Na^+,K^+-ATPase was determined.

For chemiluminescence and TBARS assays, hippocampus was homogenized in 5 volumes (1:5 w/v) of 20 mM sodium phosphate, pH 7.4, containing 140 mM KCl. For TRAP measurement, the same structure was homogenized (1:5 w/v) in 0.1 M glycine buffer, pH 8.6. For CAT and GSH-Px assays, hippocampus was homogenized (1:10, w/v)

in 10 mM potassium phosphate buffer, pH 7.6 and, for SOD activity, hippocampus was homogenized (1:10, w/v) in 50 mM Tris–HCl buffer with 1.0 mM EDTA, pH 8.2.

Met was dissolved in Tris–HCl buffer, pH 7.4 and added to the incubation medium in concentrations ranging from 0.02 to 5.0 mM.

2.3. Preparation of synaptic plasma membrane

Synaptic plasma membranes from hippocampus were prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al., 1995). These membranes were isolated using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at $69,000 \times g$ for 2 h, the fraction between 0.8 and 1.0 M sucrose interface was taken as the membrane enzyme preparation.

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

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in final volume of 200 μL. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays, as described by Wyse et al. (2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

2.5. Chemiluminescence

Chemiluminescence, representing the spontaneous light emission mainly from peroxidizing lipids, was assayed in a dark room by the method of González-Flecha et al. (1991). Incubation flasks contained 3.5 mL of medium consisting of 20 mM sodium phosphate, pH 7.4 and 140 mM KCl. The background chemiluminescence was measured for 5 min. An aliquot of 0.5 mL of hippocampus homogenate was added and chemiluminescence was measured for 10 min at room temperature. The background chemiluminescence was subtracted from the total value. Chemiluminescence was expressed as cpm/mg protein.

2.6. Thiobarbituric acid reactive substances (TBARS)

TBARS, a measure of lipid peroxidation, was determined according to Esterbauer and Cheeseman (1990). Briefly, homogenates were mixed with trichloroacetic acid 10% and thiobarbituric acid 0.67% and heated in a boiling water bath for 25 min. TBARS was determined by the absorbance at 535 nm. Results were reported as nmol of malonaldehyde per mg protein.

2.7. Total radical-trapping antioxidant potential (TRAP)

TRAP represents the total nonenzymatic antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis(2-amidinopropane) (ABAP) (Evelson et al., 2001) at room temperature. Briefly, four milliliters of 10 mM ABAP were added to the vial and the background chemilumines-cence was measured. Ten microliters of 4 mM luminol were then added and the chemiluminescence was measured. This was considered to be the initial value. Ten microliters of 80 µM trolox or homogenates (1:5 w/v in 0.1 M glycine buffer, pH 8.6) were added and chemiluminescence was measured until it reached the initial levels. The addition of trolox or tissue homogenate to the incubation medium reduces the chemiluminescence. The time necessary to return to the levels present before the addition was considered to be the induction time. The induction time is directly proportional to the antioxidant capacity of the tissue and was compared to the induction time of trolox. Results were reported as nmol of trolox per mg protein.

2.8. Catalase assay (CAT)

CAT activity was assayed by the method of Aebi (1984). H_2O_2 disappearance was continuously monitored with a spectrophotometer at 240 nm for 90 s. One unit of the enzyme is defined as 1 µmol of hydrogen peroxide consumed per minute and the specific activity was reported as units per mg protein.

2.9. Glutathione peroxidase assay (GSH-Px)

GSH-Px 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*-butyl-hydroperoxide was used as substrate. NADPH disappearance was continuously monitored with a spectrophotometer at 340 nm for 4 min. One GSH-Px unit is defined as 1 µmol of NADPH consumed per minute and specific activity was represented as units per mg protein.

2.10. Superoxide dismutase assay (SOD)

SOD activity was measured by the method of Maklund (1985). This method is based on the autoxidation of pyrogallol which is highly dependent on $O_2^{\bullet-}$. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol autoxidation and the specific activity was reported as units per mg protein.

2.11. Protein determination

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

2.12. Statistical analysis

Data were analyzed by one-way ANOVA followed by the Duncan multiple range test when *F*-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant.

3. Results

First, we investigated the effect of incubating hippo-campal homogenates in the presence of Met, at concentra-tions ranging from 0.02 to 5.0 mM, on Na^+,K^+-ATPase activity from synaptic plasma membranes of rats. In this assay, homogenates were exposed to Met and the synaptic membranes were prepared afterwards. As can be seen in Fig. 1A, 5.0 mM Met significantly inhibited Na^+,K^+-ATPase activity by approximately 25% [$F_{(5,18)} = 12.164$; $p < 0.001$].

Next, we evaluated whether the inhibitory action of Met on Na^+,K^+-ATPase activity could be mediated by oxidation of critical groups on the enzyme or by peroxidation of membrane lipids, by incubating hippocampal homogenates with 5 mM Met in the presence of the antioxidant GSH
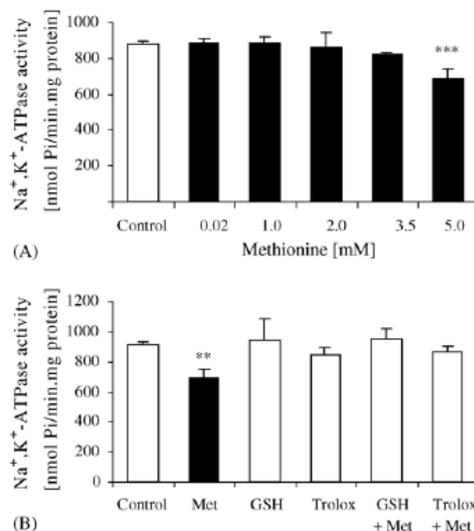
Fig. 1. Effect of hippocampal homogenates exposure to methionine (A) and to glutathione and trolox in the presence or absence of methionine (B) on Na^+,K^+-ATPase activity from synaptic plasma membrane from rats. Hip-pocampal homogenates were preincubated at 37 °C for 1 h with Met (0.02–5.0 mM), 1.0 mM GSH, 3.0 mM trolox, 5.0 mM Met plus 1.0 mM GSH or 5.0 mM Met plus 3.0 mM trolox. The synaptic plasma membranes were prepared and the enzyme activity determined. Results are expressed as means ± S.D. for four independent experiments (animals) in each group performed in duplicate. ***$p < 0.001$ and **$p < 0.01$ compared to the other groups (Duncan's multiple range test). Met – methionine.
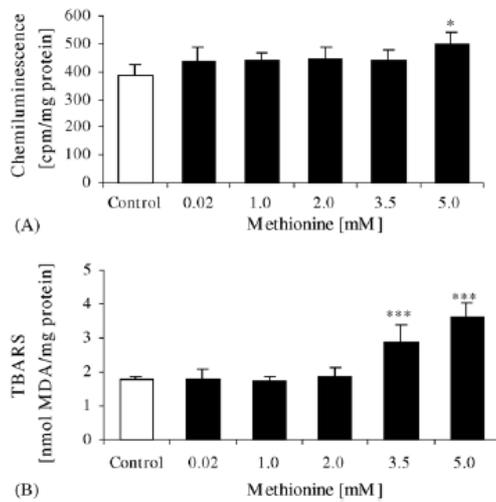
Fig. 2. Effect of methionine on chemiluminescence (A) and thiobarbituric acid reactive substances (TBARS) (B) in rat hippocampal homogenates. Results are expressed as means ± S.D. for five independent experiments (animals) in each group performed in duplicate. *$p < 0.05$ and ***$p < 0.001$ compared to control (Duncan's multiple range test).

(1.0 mM) or trolox (α-tocopherol, 3.0 mM). Post hoc analyses showed that the antioxidants per se did not alter Na$^+$,K$^+$-ATPase activity, but prevented the inhibition of the enzyme activity elicited by Met [$F(5,18) = 6.805$; $p < 0.01$] (Fig. 1B).

We also investigated the effects of different concentrations (0.02–5.0 mM) of Met on some parameters of oxidative stress, namely chemiluminescence and TBARS (indexes of lipid peroxidation), TRAP (an index of total nonenzymatic antioxidant defenses), and on the antioxidant enzymes CAT, GSH-Px and SOD in hippocampus of rats. Fig. 2A shows that 5 mM Met significantly increased chemiluminescence (∼30%) [$F(5,24) = 3.718$; $p < 0.05$]. TBARS was also significantly increased at concentrations of 3.5–5.0 mM [$F(5,24) = 31.93$; $p < 0.001$], with maximal stimulation (∼100%) occurring at 5.0 mM (Fig. 2B). Fig. 3 shows that Met significantly decreased TRAP [$F(5,24) = $
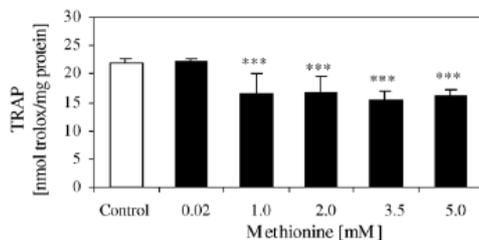
Fig. 3. Effect of methionine on total radical-trapping antioxidant potential (TRAP) in rat hippocampal homogenates. Results are expressed as means ± S.D. for five independent experiments (animals) in each group performed in duplicate. ***$p < 0.001$ compared to control (Duncan's multiple range test).

10.19; $p < 0.001$], at concentrations as low as 1.0 mM. In contrast, the activities of antioxidant enzymes were not changed by Met (data not shown).

4. Discussion

The underlying mechanisms of brain dysfunction in severe hypermethioninemia are poorly understood. In this respect, it has been demonstrated that elevated Met concentrations can be highly toxic, and that Met metabolites produced by the transamination pathway such as methanethiol might contribute to this toxicity (Smolin et al., 1981; Cooper, 1983; Benevenga and Steele, 1984). It is also known that Met has a synergistic effect on seizures elicited by homocysteine (Mudd et al., 2001). On the other hand, we have previously showed that 2.0 mM Met added to the enzymatic assay inhibits Na$^+$,K$^+$-ATPase activity from synaptic plasma membrane preparations and also reduces brain energy metabolism in rat hippocampus (Streck et al., 2002, 2003). These observations may explain the cerebral edema observed in severe hypermethioninemia since inhibition of Na$^+$,K$^+$-ATPase activity may lead to an impairment of sodium and potassium membrane transport with a consequent intracellular accumulation of sodium (Mudd et al., 2003).

In the present study we preincubated hippocampal homogenates with different concentrations of Met synaptic plasma membranes were prepared afterwards and the activity of Na$^+$,K$^+$-ATPase measured. Hippocampus was used because this structure is essential to learning/memory (Holscher, 2003; Morris et al., 2003) and hypermethioninemic patients present cognitive deficit (Mudd et al., 2000). We verified that 5 mM Met significantly inhibited (∼25%) this enzyme activity.

Considering that Na$^+$,K$^+$-ATPase is decreased by free radical formation (Lees, 1993), lipid peroxidation (Mishra et al., 1989; Viani et al., 1991) and that –SH groups of cell proteins are highly susceptible to oxidative stress (Yufu et al., 1993), we also investigated the effects of the antioxidants GSH and trolox on the inhibitory action of Met on this enzyme activity. Our results showed that GSH and trolox per se did not alter Na$^+$,K$^+$-ATPase activity, but fully prevented the inhibitory role of Met on this enzyme.

Although the exact mechanism through which Met inhibits Na$^+$,K$^+$-ATPase activity is yet unknown, the present findings suggest the involvement of reactive species probably by oxidizing SH groups of the enzyme and/or by peroxidation of membrane lipids, in which the enzyme is embedded. In this context, it should be noted that GSH acts directly as a thiol-reducing agent, as well as a scavenger of free radicals and lipid peroxidation products (Boyland and Chasseaud, 1970; Meister and Anderson, 1983; Hammond et al., 2001). In turn Vitamin E (α-tocopherol) is able to interact with cell membranes, trapping reactive oxygen species and interrupting the chain of oxidative reactions that

damage cells (Ames et al., 1993; Burton et al., 1990; Vatassery, 1998). Besides, there are studies in the literature showing that α-tocopherol can effectively slow down the progression of Alzheimer's disease and reduce the degeneration of hippocampal cells after cerebral ischemia (Hara et al., 1990; Sano et al., 1997). On the other hand, we have previously shown that Vitamins E and C prevent the reduction of Na^+,K^+-ATPase and acetylcholinesterase activities caused by hyperprolinemia and hyperargininemia (Delwing et al., 2003; Franzon et al., 2003; Bavaresco et al., 2003; Wyse et al., 2004), as well as the memory deficit caused by hyperhomocysteinemia in rats (Reis et al., 2002).

In order to evaluate whether Met induces free radical formation, we studied the effect of this amino acid on some parameters of oxidative stress in rat hippocampus. Our results showed that Met significantly increased chemiluminescence and TBARS levels, indicating that the amino acid induced lipid peroxidation. Met also provoked a decrease of TRAP, which suggest an impairment of tissue nonenzymatic antioxidant defenses, but did not change the activities of the antioxidant enzymes CAT, GSH-Px and SOD. Altogether, these findings indicate that Met, enhancing free radical production and decreasing nonenzymatic antioxidant defenses, elicited oxidative stress. Other studies demonstrated that oxidation of protein Met residues by reactive oxygen species and by transition metal-catalyzed reactions is dependent upon the presence of molecular oxygen, involving the generation of Met free radicals intermediates (Miller et al., 1996; Schöneich and Yang, 1996). In contrast, it has been demonstrated that Met exerts a protective role against free radical damage in rat brain synaptosomes (Slyshenkov et al., 2002) and there are some reports showing that the cyclic oxidation–reduction of Met residues of proteins may represent an important antioxidant mechanism (Levine et al., 1996; Moskovitz et al., 1997, 2001).

It has been proposed that alterations in Na^+,K^+-ATPase activity may represent an important neurotoxic mechanism for neurons (Lees, 1993). In this context, there are some data showing that inhibition of Na^+,K^+-ATPase activity of the similar order of magnitude as that found in our study provokes increased Na^+ uptake and cytosolic free Ca^{2+} concentrations, releasing acetylcholine and decreasing the membrane potential of synaptosomes from cerebral cortex of rats (Satoh and Nakazato, 1992). Decreased Na^+,K^+-ATPase activity leads to neuron-selective lesion in the rat brain (Lees et al., 1990) and occurs in brain of rats subjected to an experimental model of depression (Gamaro et al., 2003). Furthermore, Hattori and colleagues found a reduction (23%) of Na^+,K^+-ATPase activity in the brain of patients of Alzheimer's disease, and suggested that the decreased enzyme activity could be involved in the pathophysiology of this neurodegenerative disorder (Hattori et al., 1998).

Although it is difficult to extrapolate our in vitro data to the human condition, it is tempting to especulate that neurological symptoms observed in hypermethioninemia may be related to high tissue concentrations of Met having an adverse effect on brain function through oxidative stress and inhibition of Na^+,K^+-ATPase activity. However, whether these or other abnormalities are the main factors responsible for the brain damage in hypermethioninemia remains to be elucidated.

Acknowledgements

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CAPÍTULO II – ARTIGO 2

Chemically induced model of hypermethioninemia in rats

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Chemically induced model of hypermethioninemia in rats

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Abstract

In the present study, we developed a chronic chemically induced model of hypermethioninemia in rats. We induced elevated concentrations of methionine in the blood by injecting subcutaneously methionine (1.34–2.68 μmol/g of body weight) to developing animals of various ages. Brain methionine concentrations were approximately 1.25 μmol/g wet tissue (∼1.0 mM). We then injected the same doses of methionine to young rats twice a day at 8 h intervals from the 6th to the 28th postpartum day. Controls received saline in the same volumes. The body, brain and hippocampus of rats were weighed after treatment and showed that hypermethioninemic animals had no differences in these parameters, when compared to the control group, suggesting that methionine did not cause malnutrition in the rats. Considering that experimental animal models are useful to understand the pathophysiology of human disease, the present model of hypermethioninemia may contribute to the investigation of the mechanisms of brain damage caused by high tissue methionine levels.
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Keywords: Hypermethioninemia; Metabolic disease; Animal model; Methionine

1. Introduction

Methionine (Met) is an essential amino acid important for the normal growth and development of mammals (Finkelstein and Martin, 1986). However, if the concentration of Met increases, it may become toxic (Benevenga and Steele, 1984; Cooper, 1983; Mudd et al., 2001; Smolin et al., 1981). In this context, high plasma levels of Met (hypermethioninemia) have been encountered in various inherited disorders such as methionine adenosyltransferase deficiency (Chamberlin et al., 1996; Mudd et al., 2000, 2001), homocystinuria (Mudd et al., 2001) and glycine N-methyltransferase deficiency in which neurological symptoms may occur (Augoustides-Savvopoulou et al., 2003; Ozias and Schalinske, 2003). In addition, extreme elevations of adenosylhomocysteine and adenosylmethionine occur in adenosylhomocysteine hydrolase deficiency (Baric et al., 2005).

Despite a great deal of works on the neurotoxic effects of Met, the mechanisms behind these actions remain to be

elucidated. However, *in vitro* studies have begun to identify some of the actions of Met to induce brain damage. In this context, we have previously demonstrated that this amino acid induces oxidative stress, reduces brain energy metabolism and inhibits Na^+,K^+-ATPase activity in rat hippocampus *in vitro* (Stefanello et al., 2005; Streck et al., 2002a, 2003). These alterations may explain the cerebral edema observed in patients with methionine adenosyltransferase deficiency since reduction of Na^+,K^+-ATPase caused by hypermethioninemia may lead to an impairment of sodium and potassium membrane transport with a consequent intracellular accumulation of sodium and water (Mudd et al., 2003). Interestingly, clinical studies demonstrated that Met levels are elevated in cerebrospinal fluid of psychotic patients (Regland et al., 2004).

Animal experimental models can be used to better understand the pathophysiology of human diseases. In this context, our laboratory has developed animal models for some inherited metabolic disorders, such as phenylketonuria (Wyse et al., 1994, 1995), hyperprolinemia type II (Moreira et al., 1989), homocystinuria (Streck et al., 2002b), methylmalonic and propionic acidemias (Brusque et al., 1999; Dutra et al., 1991). In these models, we reproduced the peak levels achieved by the

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accumulating metabolites after feedings by repeated injections to the rats during a critical period of brain development (6th to the 28th postnatal day).

In the present study we attempted to create a chemically induced experimental model of hypermethioninemia in young rats by injecting Met twice a day from the 6th to the 28th post-partum day.

2. Materials and methods

2.1. Animals

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Science of Health, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12 h light/dark cycle in an air-conditioned constant temperature ($22 \pm 1\,^{\circ}$C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

2.2. Chemicals

Ortho-phthaldialdehyde (OPA), methionine standard and homocysteic acid were obtained from Sigma Chem. Co. (St. Louis, MA, USA), whereas mercaptoethanol and methanol for chromatography was purchased from Merck (Darmstadt, Germany).

2.3. Tissue preparation

Wistar rats of 6–13, 14–20 and 21–28 days of life received a subcutaneous injection of Met. Blood was collected by cardiac puncture at 15 min, 1, 2, 8 or 12 h after Met injection, and the plasma was separated. The animals were sacrificed and the brain was immediately isolated. The olfactory bulbs, pons, medulla and the cerebellum were discarded and the cerebrum was homogenized in five volumes of saline solution. Plasma and cerebrum homogenates were deproteinized with methanol (1:5, v/v) and centrifuged at $400 \times g$ for 10 min. The supernatants were removed for Met determination.

2.4. Methionine quantification

The concentrations of Met in plasma and cerebrum were determined by high-performance liquid chromatography (HPLC) according to Joseph and Marsden (1986). The analysis was performed using a reverse phase column (ODS 25 cm × 4.6 mm × 5 μm) and fluorescent detection after pre-column derivatization with OPA plus mercaptoethanol. The flow rate was adjusted to 1.4 mL/min in a gradient of the mobile phase of methanol and 0.5 M sodium phosphate buffer pH 5.5 (buffer A, 80% methanol; buffer B, 20% methanol). Each sample run lasts 45 min. Met was identified by its retention time

Table 1
Pharmacokinetic parameters of methionine and doses administered according to rat age

Age (days)	$t_{1/2}$ (min)	V_d (mL/g)	Cl_p (μL/min g)	Met doses (μmol/g weight)
6–13	302.4	0.90	2.07	1.34
14–20	206.9	0.96	3.22	2.01
21–28	132.8	0.92	4.80	2.68

Note: The parameters were calculated after acute subcutaneous injections of Met, after which animals were sacrificed at regular time intervals (15 min, 1, 2, 8 and 12 h). $t_{1/2}$: plasmatic half-time; V_d: apparent volume of distribution; Cl_p: plasmatic clearance; Met: methionine.

and was quantitatively determined by using its chromatographic peak area and correlating with the internal standard peak area (homocysteic acid).

2.5. Chemically induced hypermethioninemia

Doses of Met administered were chosen in order to induce high plasma Met levels similar to those found in patients affected by some conditions with hypermethioninemia. The chosen Met doses were 1.34–2.68 μmol/g of body weight depending on animal age, as shown in Table 1. We then determined the following pharmacokinetic parameters, apparent volume of distribution (V_d), plasmatic half-time ($t_{1/2}$) and plasmatic clearance (Cl_p) after Met injection. Chronically induced hypermethioninemia was also used in the present study by administering Met subcutaneously twice a day with an interval of 8 h between injections to rats from the 6th to the 28th day of life. Control rats received saline solution in the same volumes. Twelve hours after the last injection, the body, cerebrum and hippocampus of rats were weighed.

2.6. Statistical analysis

Data were analyzed by the Student's *t*-test or by one-way ANOVA followed by Duncan multiple range test when *F*-value was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant.

3. Results

Fig. 1 shows that 15 min after injection plasma Met levels were about 30-fold higher (approximately 2 mM) than normal values [6–13 days: ($F_{(5,12)} = 67.19$, $p < 0.001$); 14–20 days: ($F_{(5,12)} = 161.05$, $p < 0.001$); 21–28 days: ($F_{(5,12)} = 331.32$, $p < 0.001$)]. Fig. 2 shows that cerebral Met concentrations were approximately 15-fold higher (around to 1.25 μmol/g wet tissue) normal concentrations 60 min after injection [6–13 days: ($F_{(5,12)} = 135.06$, $p < 0.001$); 14–20 days: ($F_{(5,12)} = 131.14$, $p < 0.001$); 21–28 days: ($F_{(5,12)} = 44.96$, $p < 0.001$).

The pharmacokinetic parameters apparent volume of distribution (V_d), plasmatic half-time ($t_{1/2}$) and plasmatic clearance (Cl_p) were determined after Met injection (Table 1). It can be
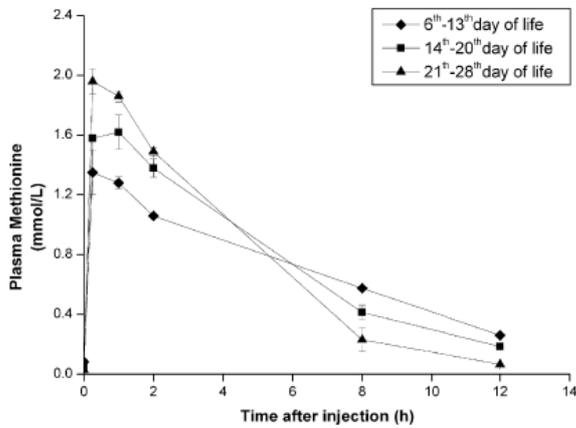
Fig. 1. Methionine plasma levels following methionine administration according to the age of rats. Rats of 6–13, 14–20 and 21–28 days of age were administered methionine and the blood was collected 15 min, 1, 2, 8 and 12 h after injection. Data are expressed as mean ± S.E.M. for three animals in each group (ANOVA).

observed in the table that higher doses of Met were necessary to achieve maximal plasma Met concentration as animal age advanced.

Taking these data into consideration, we developed a chronic chemical model with hypermethioninemia by injecting a buffered Met solution twice a day at 8 h intervals from the 6th to the 28th day of life, corresponding to the most vulnerable period of brain development in the rat. Control rats received saline in the same volumes. As can be seen in Table 2, body, cerebrum and hippocampus weight of rats submitted to chronic Met administration did not differ from saline-treated rats, suggesting that Met did not cause malnutrition in the animals. However, we cannot assume that brain damage did not occur in these animals, so that neurochemical parameters should be investigated in order
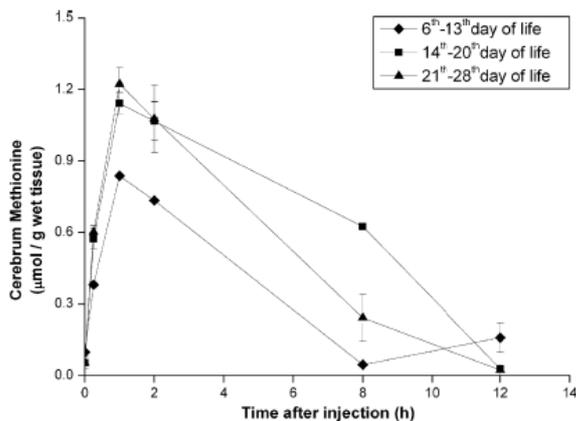

Fig. 2. Methionine cerebrum concentrations following methionine administration according to the age of rats. Rats of 6–13, 14–20 and 21–28 days of age were sacrificed after 15 min, 1, 2, 8 and 12 h and the brain was immediately separated and homogenized. Data are expressed as mean ± S.E.M. for three animals in each group (ANOVA).

Table 2
Effect of chemically induced hypermethioninemia on body, cerebrum and hippocampus weight of rats

	Treatment	
	Saline	Methionine
Body weight (g)	73.25 ± 4.80	69.01 ± 4.10
Cerebrum weight (g)	1.10 ± 0.05	1.07 ± 0.02
Hippocampus weight (g)	0.10 ± 0.01	0.10 ± 0.01

Note: Data are expressed as mean ± S.D. for eight rats per group. No significant difference between groups was detected (unpaired Student's *t*-test).

to clarify the chronic effects of high sustained levels of Met in the brain.

4. Discussion

The principal objective of the present study was to produce high sustained levels of methionine in plasma of rats similar to those found in some inherited pathological conditions in which the concentrations of this amino acid are increased (Augoustides-Savvopoulou et al., 2003; Mudd et al., 2001; Ozias and Schalinske, 2003). Concentrations of Met approximately 30-fold the normal levels (controls) were achieved in plasma by subcutaneous administration of this amino acid. The maximal plasma Met concentrations were reached 15 min after a single injection of Met, gradually decreasing afterwards, but remained high for at least 6 h. Brain Met concentrations were maximal 60 min after injection, and corresponded to approximately 15-fold the normal values. Brain Met concentrations also decreased along time, but remained relatively high even 8 h after injection. Since relatively high concentrations of Met were encountered in the brain, it can be presumed that Met crosses the blood-brain barrier, corroborating with other data showing that Met is transported via the L-carrier system (Kracht et al., 2003; O'Kane and Hawkins, 2003).

The pharmacokinetic parameters measured after Met administration showed that plasmatic clearance increased and plasmatic half-time decreased as animal age advanced. These data probably indicate that renal excretion and hepatic metabolism of Met are not fully developed in very young rats. Additionally, we observed that the concentrations of Met in the brain decreased more slowly than those in the plasma, remaining relatively high up to 8 h after Met injection. It should be, however, emphasized that, besides Met, other metabolites such as *S*-adenosylmethionine, homocysteine and *S*-adenosylhomocysteine, perhaps cystathionine and sarcosine probably increased in the plasma of rats injected with Met. Although the extent to which such changes occur in brain is uncertain, it is unlikely that they have contributed decisively to the effects observed in the present study.

We then administered chronically twice a day the same doses of Met utilized to calculate the pharmacokinetic parameters during a period of rapid brain development (from the 6th to the 28th postnatal day of life) in which crucial events occur, such as synaptogenesis (Clark et al., 1993), myelogenesis and intense neural proliferation (Enesco and Leblond, 1962; Winick and

Noble, 1965). Our findings demonstrated that animals submitted to this model had no differences in body, cerebrum or hippocampus weight, when compared to the control group, indicating that Met does not cause malnutrition in the rats, which could otherwise interfere with some important studies in the brain. Therefore, this chemical model seems suitable for futures studies aiming to investigate the role of high brain Met concentrations on neurochemical parameters and behavior in order to clarify the neuropathological changes observed in hypermethioninemic patients. However, it should be taken into consideration that Met derivatives may also increase following Met administration so that data originated from this model should be interpreted with caution.

Acknowledgements

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CAPÍTULO III – ARTIGO 3

Effect of hypermethioninemia on some parameters of oxidative stress and on Na⁺,K⁺-ATPase activity in hippocampus of rats

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ORIGINAL PAPER

Effect of hypermethioninemia on some parameters of oxidative stress and on Na^+,K^+-ATPase activity in hippocampus of rats

Francieli M. Stefanello · Emilene B. S. Scherer ·
Andréa G. Kurek · Cristiane B. Mattos ·
Angela T. S. Wyse

Abstract In the present study we investigated the effect of chronic administration of methionine, a metabolite accumulated in many inherited pathological conditions such as methionine adenosyltransferase deficiency and homocystinuria, on some parameters of oxidative stress, namely thiobarbituric acid reactive substances (TBARS), catalase activity and total thiol content, as well as on Na^+,K^+-ATPase activity in rat hippocampus. For chronic treatment, rats received subcutaneous injections of methionine (1.34–2.68 μmol/g of body weight), twice a day, from the 6th to the 28th day of age and controls received saline. Animals were killed 12 h after the last injection. Results showed that chronic hypermethioninemia significantly increased TBARS, decreased Na^+,K^+-ATPase activity but did not alter catalase and total thiol content. Since chronic hypermethioninemia altered TBARS and Na^+,K^+-ATPase activity at 12 h after methionine administration, we also investigated the effect of acute administration of this amino acid on the same parameters studied after chronic methionine administration. For acute treatment, 29-day-old rats received one single injection of methionine (2.68 μmol/g of body weight) or saline and were killed 1, 3 or 12 h later. Results showed that rats subjected to acute hypermethioninemia presented a reduction of Na^+,K^+-ATPase activity and an increase in TBARS when the animals were killed at 3 and 12 h, but not at 1 h, after methionine administration. These data indicate that hypermethioninemia increases lipid peroxidation which may, at least partially, explain the effect of methionine on the reduction in Na^+,K^+-ATPase activity. If confirmed in human beings, our findings could suggest that the induction of oxidative stress and the inhibition of Na^+,K^+-ATPase activity caused by methionine might contribute to the neurophysiopathology observed in patients with severe hypermethioninemia.

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Introduction

Tissue accumulation of methionine (Met) can occur in many metabolic disorders, such as methionine adenosyltransferase deficiency and homocystinuria. Affected patients with these disorders present a variable degree of neurological dysfunction, including mental retardation, cognitive deficit and cerebral edema; however the exact mechanisms involved in these alterations remain poorly understood (Mudd *et al.* 2000, 2001).

Accumulating evidence suggests that reactive oxygen species and/or oxidative stress can contribute to neurological dysfunction present in many neurodegenerative disorders, including Alzheimer's disease (Bergendi *et al.* 1999; Mancuso *et al.* 2006; Halliwell 2006). At this point, it should be emphasized that lipid, protein and DNA oxidative damage, as well as reduced concentrations of enzymatic and nonenzymatic defenses have been demonstrated in patients affected by these disorders (Olanow 1993; Perry *et al.* 2003; Halliwell 2006). In this respect, we have shown that Met in vitro induces oxidative stress in rat hippocampus (Stefanello *et al.* 2005).

Na$^+$,K$^+$-ATPase is an enzyme embedded in the plasma membrane that catalyzes the active transport of monovalent cations inside and outside the cell membrane at the expense of ATP hydrolyzing. This enzyme is responsible for generating and maintaining membrane potential necessary for neuronal excitability and thus disturbances in its activity could have grave consequences for central nervous system (CNS) functioning (Erecinska and Silver 1994; Mobasheri *et al.* 2000). Accordingly, there are reports showing that Na$^+$,K$^+$-ATPase activity is reduced in epilepsy (Grisar *et al.* 1992), depressive disorders (Goldstein *et al.* 2006), an experimental model of cerebral ischemia (Wyse *et al.* 2000), a depression model (de Vasconcellos *et al.* 2005), and in many neurodegenerative disorders, including Alzheimer's disease (Hattory *et al.* 1998; Vignini *et al.* 2006). On the other hand, it has been demonstrated that free radicals and/ or oxidative stress inhibit Na$^+$,K$^+$-ATPase activity (Lees 1993; Jamme *et al.* 1995; Rauchová *et al.* 1999). In addition, we have shown that Met inhibits hippocampal Na$^+$, K$^+$-ATPase activity in vitro (Streck *et al.* 2002; Stefanello *et al.* 2005).

Therefore, considering that oxidative stress and Na$^+$,K$^+$-ATPase activity are associated with neurological dysfunction (Mobasheri *et al.* 2000; Halliwell 2006; Vignini *et al.* 2006) and Met alters these parameters in vitro (Stefanello *et al.* 2005), in the present study we investigated the effect of chronic administration of Met on some parameters of oxidative stress, namely thiobarbituric acid reactive substances (TBARS), catalase activity and total thiol (SH) content, as well as on Na$^+$,K$^+$-ATPase activity in synaptic plasma membrane from hippocampus of rats. The Na$^+$, K$^+$-ATPase activity and TBARS levels were also tested in hippocampus of rats subjected to acute hypermethioninemia. The working hypothesis is that the administration of Met could induce oxidative stress and this alteration would be associated with a possible inhibition of Na$^+$,K$^+$-ATPase activity. Hippocampus was used because this cerebral structure is essential to learning/memory (Holscher 2003;

Morris *et al.* 2003) and patients with severe hypermethioninemia present impairment in memory (Mudd *et al.* 2000, 2001).

Materials and methods

Subjects and reagents

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Science of Health, Federal University of 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 ($22\pm1°C$) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. The chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Methionine administration procedure

For chronic treatment, Met solution was administered subcutaneously twice a day at 8 h intervals from the 6th to 28th day of age. Control animals received saline solution in the same volumes as those applied to Met-treated rats. Met doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Stefanello *et al.* 2007). Animals received 1.34 µmol Met/g body weight during the first 8 days of treatment, 2.01 µmol Met/g body weight from days 14th to 20th, and 2.68 µmol Met/g body weight from the 21st to 28th day. Rats subjected to this treatment achieved plasma Met levels (around to 2 mmol/l) similar to those found in hypermethioninemic patients with some inherited pathological conditions (Mudd *et al.* 2001; Augoustides-Savvopoulou *et al.* 2003).

Maximal brain levels were achieved 60 min after Met administration (1.25 µmol/g wet tissue). Twelve hours after treatment, plasma and brain Met concentrations returned to normal levels. The animals were killed 12 h after the last injection and hippocampus were quickly dissected out. For acute treatment, 29-day-old rats received one single subcutaneous injection of Met (2.68 µmol/g of body weight) or saline (control) and were killed 1, 3 or 12 h later. For both treatments, Met was dissolved in 0.9% NaCl solution and buffered to pH 7.4.

Tissue and homogenate preparation

Animals were killed by decapitation without anesthesia, the brain was quickly removed and the hippocampus was dissected. For TBARS and total thiol content assays, hippocampus was homogenized in ten volumes (1:10, w/v) of 1.15% KCl or in phosphate buffer saline (PBS), pH 7.5, containing 1 mM EDTA, respectively. For CAT assay, hippocampus was homogenized (1:10, w/v) in 10 mM potassium phosphate buffer, pH 7.6. For Na^+,K^+-ATPase assay, hippocampus was homogenized
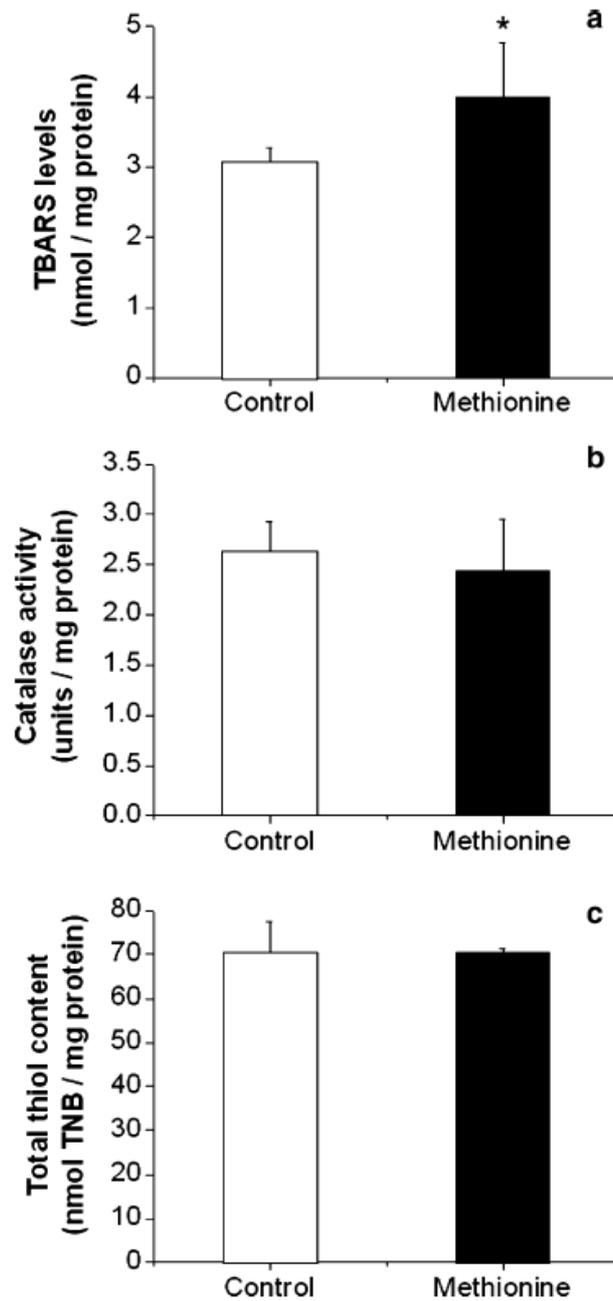
Fig. 1 Effect of chronic administration of methionine on thiobarbituric acid reactive substances (TBARS) (a), catalase activity (b) and total thiol content (c) in hippocampus of rats. Data are expressed as mean±SD of 4–6 animals in each group. *$p<0.05$ compared to control groups (Student's t test)

in ten volumes (1:10, w/v) of 0.32 M sucrose solution containing 5.0 mM HEPES and 0.1 mM EDTA, pH 7.4. After homogenization, synaptic plasma membranes were prepared and the activity of Na^+,K^+-ATPase was determined.

TBARS assay

TBARS, an index of lipid peroxidation, were determined according to the method described by Ohkawa *et al.* (1979). Briefly, 50 μl of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid were added to 500 μl of tissue homogenate in a Pyrex tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifuged at $1,000 \times g$ for 10 min. The organic layer was taken and the resulting pink color was determined in a spectrophotometer at 535 nm. The results were reported as nmol of TBARS per mg protein.

Catalase assay

Catalase activity was assayed by the method of Aebi (1984). H_2O_2 disappearance was continuously monitored with a spectrophotometer at 240 nm for 90 s. One unit of the enzyme is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity was reported as units per mg protein.

Total thiol content assay

Total thiol content was determined using the DTNB method, as described by Aksenov and Markesbery (2001) with some modifications. Briefly, 50 μl of the sample was mixed with 980 μl of PBS, pH 7.5, containing 1 mM EDTA. The reaction was started by the addition of 30 μl of 10 mM DTNB stock solution in PBS.
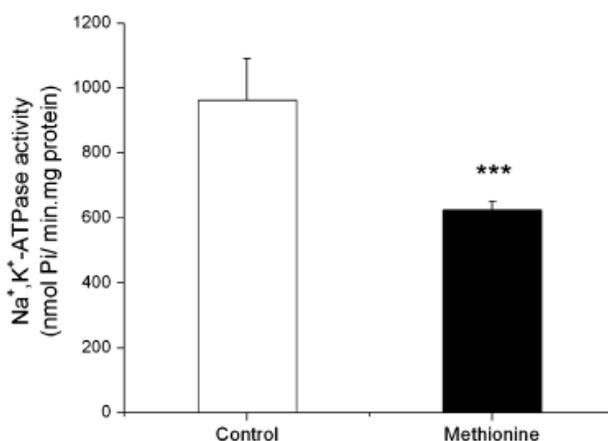
Fig. 2 Effect of chronic administration of methionine on Na^+,K^+-ATPase activity from synaptic plasma membrane of rat hippocampus. Data are expressed as mean±SD of five animals in each group. ***$p<0.001$ compared to control groups (Student's *t* test)

After 30 min of incubation at room temperature, the absorbance at 412 nm was measured and the amount of TNB formed was calculated. The results were reported as nmol of TNB per mg protein.

Preparation of synaptic plasma membrane

Synaptic plasma membranes from hippocampus were prepared according to the method of Jones and Matus (1974) with some modifications (Wyse *et al.* 1995). These membranes were isolated using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000 × *g* for 2 h, the fraction between 0.8 and 1.0 M sucrose interface was taken as the membrane enzyme preparation.

Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris-HCl, pH 7.4, in final volume of
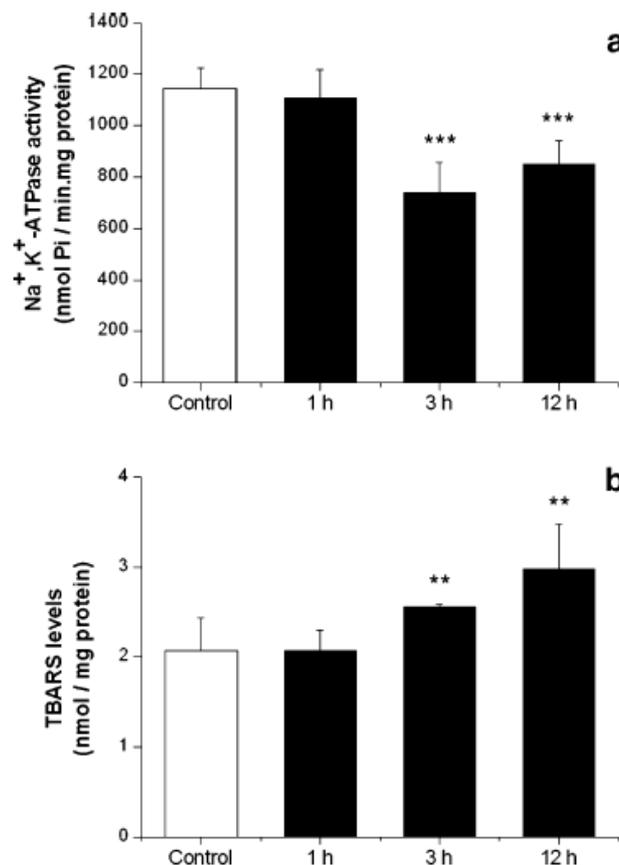
Fig. 3 Effect of acute administration of methionine on Na^+,K^+-ATPase activity (**a**) and on thiobarbituric acid reactive substances (TBARS) (**b**) in hippocampus of rats. Data are expressed as mean±SD of 4–5 animals in each group. **$p < 0.01$; ***$p < 0.001$ compared to control groups (Duncan's multiple range test)

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200 μl. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays, as described by Wyse *et al.* (2000). Released inorganic phosphate (Pi) was measured by the method of Chan *et al.* (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

Protein determination

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

Statistical analysis

Data were analyzed by Student's *t* test or by one-way analysis of variance (ANOVA) followed by the Duncan's multiple range test when *F*-value was significant. 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

First, we verified the effect of chronic administration of Met on some parameters of oxidative stress in rat hippocampus. Figure 1 shows that chronic hypermethioninemia significantly increased (30%) TBARS values (a) $[t(10) = -2.83; p < 0.05]$ when compared to control group (saline). As can be also observed in Fig. 1, Met did not alter catalase activity (b) $[t(6) = 0.55; p > 0.05]$ and total thiol content (c) $[t(6) = 0.005; p > 0.05]$.

Next, we determined the activity of Na^+,K^+-ATPase in synaptic plasma membranes from hippocampus of rats subjected to chronic hypermethioninemia. Figure 2 shows that Met-treated rats presented a significant reduction (35%) in Na^+,K^+-ATPase activity $[t(8) = 5.71; p < 0.001]$ when compared to control groups. Since chronic administration of Met to developing rats (6th to the 28th postpartum day) provoked changes in TBARS values and Na^+,K^+-ATPase activity when the animals were killed at 12 h after the last injection (chronic studies), we also evaluated these same parameters in hippocampus of young rats (29-day-old) at different times (1, 3 or 12 h) after the administration of Met (acute studies). Figure 3 shows that acute hypermethioninemia significantly decreased (~30%) Na^+,K^+-ATPase activity (a) $[F(3,15) = 18.86; p < 0.001]$ and increased (~35%) TBARS (b) $[F(3,15) = 8.57; p < 0.01]$ at 3 and 12 h, but not 1 h, after Met injection.

Discussion

Neurological dysfunction is observed in patients with severe hypermethioninemia (Mudd *et al.* 2000, 2001), whose physiopathology is still poorly understood.

However, there is some data in the literature suggesting that elevated Met concentrations and/or its metabolites are potentially toxic (Benevenga and Steele 1984; Mudd *et al.* 2001; Garlick 2006). In this context, we have demonstrated that Met in vitro reduces energy metabolism, induces oxidative stress and inhibits Na^+,K^+-ATPase activity in rat hippocampus (Streck *et al.* 2002, 2003; Stefanello *et al.* 2005). It has also shown that Met increases hippocampal acetylcholinesterase activity in vitro (Schulpis *et al.* 2006). On the other hand, clinical studies have demonstrated that Met levels are elevated in cerebrospinal fluid of psychotic patients (Regland *et al.* 2004).

Animal models are useful to better understand the pathophysiology of diseases. In this context, we have recently developed in our laboratory a chemically experimental model of hypermethioninemia (Stefanello *et al.* 2007), in which we produced high levels of Met in blood of rats similar to those found in some pathological conditions such as methionine adenosyltransferase deficiency, homocystinuria and glycine *N*-methyltransferase deficiency (Mudd *et al.* 2001; Augoustides-Savvopoulou *et al.* 2003). The drug was administered during a period characterized by intense synaptogenesis and gliogenesis and when cerebral structures involved in cognition have a rapid development in rats (Loo *et al.* 1980; Davis and Squire 1984; Clark *et al.* 1993; Miller *et al.* 1994). Animals exposed to Met treatment presented no differences in physical growth and brain weight when compared to the control group, suggesting that Met did not cause malnutrition in the rats, which could otherwise interfere with some important studies in the brain (Stefanello *et al.* 2007). By using this model, we initially evaluated the effect of chronic Met administration on some parameters of oxidative stress, namely TBARS, catalase activity and total thiol content, as well as on Na^+,K^+-ATPase activity in rat hippocampus at 12 h after the last Met injection. We showed that chronic hypermethioninemia significantly increased TBARS, but did not alter catalase and total thiol content. Considering that TBARS reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge 2006), our data indicate that Met increased spontaneous lipid peroxidation in the brain, inducing oxidative damage in membrane lipids. We also demonstrated that Met administration significantly decreased Na^+,K^+-ATPase activity from synaptic plasma membranes from rat hippocampus. These findings are in agreement with previous in vitro data from our laboratory showing that Met induces oxidative stress and inhibits brain Na^+,K^+-ATPase activity (Stefanello *et al.* 2005).

According to the pharmacokinetic parameters determined by Stefanello *et al.* (2007), the Met concentration decreases after 6 h of administration and returned to normal levels after 12 h. Here presented results indicate that high levels of Met are not necessary to produce inhibition of Na^+,K^+-ATPase activity suggesting that this effect is rather indirect. In this context, numerous studies have demonstrated the vulnerability of membrane bound Na^+,K^+-ATPase to free radicals (Jamme *et al.* 1995; Rauchová *et al.* 1999). The oxidation of sulfhydryl or other essential groups of the enzyme, besides the damage to membrane phospholipids by the peroxidative process and the associated alteration of fluidity or other membrane properties have been implicated in the inactivation of Na^+,K^+-ATPase (Yufu *et al.* 1993; Fleuranceau-Morel *et al.* 1999; Lehtosky *et al.* 1999). Accordingly, our recent data showed that the antioxidants such as glutathione (GSH), SH-group protecting agent, and trolox (α-tocopherol) prevented in vitro the inhibitory effect of Met on cerebral

Na$^+$,K$^+$-ATPase activity in rats (Stefanello *et al.* 2005). Considering that chronic hypermethioninemia altered TBARS values and Na$^+$,K$^+$-ATPase activity at 12 h after Met administration, we also determined the effect of acute hypermethioninemia on these same parameters in hippocampus of rats at 1, 3 or 12 h after Met injection. Our results demonstrated that this amino acid, at 3 and 12 h after administration, significantly decreased Na$^+$,K$^+$-ATPase activity and increased TBARS. Although not conclusive, the present in vivo findings suggest that lipid peroxidation may, at least partially, explain the inhibition provoked by Met towards Na$^+$,K$^+$-ATPase activity in the hippocampus. Na$^+$,K$^+$-ATPase activity is critical for maintaining the basal membrane potential necessary for a normal neurotransmission, and alterations in this enzyme activity may be a link between many common neurotoxic mechanisms in neurons (Lees and Leong 1995; Wang *et al.* 2003; Yu 2003). In this context, neuronal death associated with failure of the Na$^+$, K$^+$ pump may lead to apoptosis and necrosis mediated by depletion of K$^+$ and accumulation of Na$^+$ and Ca^{2+}, respectively (Xiao *et al.* 2002). On the other hand, inhibition of Na$^+$,K$^+$-ATPase has been also associated with excitotoxicity and neurodegenerative disorders (Lees 1993; Cousin *et al.* 1995; Pisani *et al.* 2006; Vignini *et al.* 2006).

Therefore, although it is difficult extrapolate our results to the condition human, we cannot rule out a possible correlation between an inhibition of Na$^+$,K$^+$-ATPase activity and induction of oxidative stress caused by Met and the brain damage present in hypermethioninemic patients. However, more studies are necessary to investigate additional mechanisms involved in severe hypermethioninemia.

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CAPÍTULO IV – ARTIGO 4

Reduction of gangliosides, phospholipids and cholesterol content in cerebral cortex of rats caused by chronic hypermethioninemia

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Reduction of gangliosides, phospholipids and cholesterol content in cerebral cortex of rats caused by chronic hypermethioninemia

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Abstract

Neurological dysfunction is observed in patients with severe hypermethioninemia, whose physiopathology is still poorly understood. In the current study we investigated the effect of chronic administration of methionine on the content and species of gangliosides and phospholipids, as well as on the concentration of cholesterol in rat cerebral cortex. Wistar rats received subcutaneous injections of methionine (1.34–2.68 μmol/g of body weight), twice a day, from the 6th to the 28th day of age and controls received saline. Animals were killed 12 h after the last injection. Results showed that methionine administration significantly decreased the total content of lipids in cerebral cortex of rats. We also observed that this amino acid significantly reduced the absolute quantity of the major brain gangliosides (GM1, GD1a, GD1b and GT1b) and phospholipids (sphingomyelin, phosphatidylcholine and phosphatidylethanolamine). We also showed that Na^+,K^+-ATPase activity and TBARS were changed in cerebral cortex of rats subjected to hypermethioninemia. If confirmed in human beings, these data could suggest that the alteration in lipid composition, Na^+,K^+-ATPase activity and TBARS caused by methionine might contribute to the neurophysiopathology observed in hypermethioninemic patients.
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Keywords: Hypermethioninemia; Gangliosides; Phospholipids; Cholesterol; Cerebral cortex

1. Introduction

Tissue accumulation of methionine (Met) is a biochemical hallmark of many inherited metabolic disorders, such as methionine adenosyltransferase (MAT) deficiency and homocystinuria. Although, some patients with MAT deficiency did not present significant clinical symptoms, a significant number of affected patients present neurological manifestations, including cognitive deficit, mental retardation, cerebral edema and demyelination (Chamberlin et al., 1996; Mudd et al., 2000, 2001).

It has been shown that lipids, such as gangliosides, phospholipids and cholesterol present a variety of important physiological functions in neurons (Agranoff and Hajra, 1994). Gangliosides, a complex family of sialylated glycosphingoli-pids, are particularly abundant in the neuronal membranes acting on proliferation and neuronal differentiation, myelination and synaptic transmission (Ando, 1983; Tettamanti and Riboni, 1994; Nagai, 1995; Kolter et al., 2002; Moccheti, 2005). It has also been proposed that gangliosides may play significant role in learning/memory mechanisms (Rahmann, 1995; She et al., 2005). Phospholipids are a heterogeneous group of compounds that constitute the backbone of neural membranes. They provide the membrane with suitable environment, fluidity and ion permeability, and are required for the function of integral membrane proteins, receptors and ion channels (Farooqui et al., 2004). Cholesterol is a structural component of membranes and is required for viability and cellular proliferation (Ohvo-Rekilä et al., 2002). It and glycosphingolipids are concentrated in detergent-resistant microdomains or lipid rafts, which are seen as platforms for the signal transduction events (Paratcha and Ibanez, 2002). Cholesterol is also involved in membrane trafficking, myelin formation and synaptogenesis (Mauch et al., 2001; Valenza and Cattaneo, 2006).

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There are some evidences showing that alterations in the content and composition of gangliosides occur in experimental models of organic acidemia (Wajner et al., 1988; Brusque et al., 1998; Trindade et al., 2002), hypoxia/ischemia (Trindade et al., 2001; Ramirez et al., 2003) and Alzheimer's disease (Barrier et al., in press). Additionally, it has been demonstrated that brain phospholipid and cholesterol contents decrease with advancing age (Svennerholm et al., 1994) and in patients with neurodegenerative diseases (Svennerholm and Gottfries, 1994; Farooqui et al., 2004).

The aim of this study was to investigate whether early chronic administration of Met would affect the content and the species of gangliosides and phospholipids, as well as the concentration of cholesterol in cerebral cortex of rats. Additionally, considering that Na^+,K^+-ATPase and thiobarbituric acid-reactive substances (TBARS) are marker of neuronal membrane (Jones and Matus, 1974) and lipid peroxidation (Ohkawa et al., 1979), respectively, we also evaluated the effect of chronic hypermethioninemia on these parameters in cerebral cortex of rats. Our hypothesis is that hypermethioninemia could alter lipid content, Na^+,K^+-ATPase activity and lipid peroxidation in brain of rats. We used cerebral cortex because this structure is involved in memory/learning mechanisms (Izquierdo et al., 1998) and several patients with hypermethioninemia present cognitive impairment (Mudd et al., 2000, 2001).

2. Experimental procedures

2.1. Subjects and reagents

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Science of Health, Federal University of 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.

Silica-gel 60 thin-layer chromatography (TLC) sheets were supplied by Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma–Aldrich (Saint Louis, MO, USA) and the solvents used were of analytical grade.

2.2. Methionine administration procedure

Methionine was dissolved in 0.9% sodium chloride solution and was adjusted to pH 7.4. For the chronic treatment, Met solution was administered subcutaneously twice a day at 8 h intervals from the 6th to 28th day of age. Control animals received saline solution in the same volumes as those applied to Met-treated rats. Met doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Stefanello et al., 2007a). Animals received 1.34 μmol Met/g body weight during the first 8 days of treatment, 2.01 μmol Met/g body weight from day 14th to 20th, and 2.68 μmol Met/g body weight from day 21st to the 28th. Rats subjected to this treatment achieved plasma Met levels (2 mmol/L) similar to those found in hypermethioninemic patients with some inherited pathological conditions (Mudd et al., 2001; Augoustides-Savvopoulou et al., 2003). Maximal brain levels were achieved 60 min after Met administration (1.25 μmol/g wet tissue). Twelve hours after treatment, plasma and brain Met concentrations returned to normal levels. The animals were killed 12 h after the last injection and cerebral cortex was quickly dissected out.

2.3. Lipid extraction

The cerebral cortex was weighed and homogenized in a 2:1 mixture of chloroform:methanol (C:M, 2:1, v/v) to a 20-fold dilution of tissue mass and centrifuged at $800 \times g$ for 10 min. The pellet was re-homogenized in C:M (1:2) to a 10-fold dilution of original sample mass (Folch et al., 1957). The C:M extracts were combined and this pool was used for the following determinations.

2.4. Total gangliosides, phospholipids and cholesterol determinations

Aliquots from the total lipid extracts were used for ganglioside determination by the N-acetyl-neuraminic acid (NeuAc) quantification with the thiobarbituric acid assay described by Skoza and Mohos (1976).

Phospholipid and cholesterol were quantified in aliquots from total lipid extracts according to the method of Bartlett (1959) and to the Trinder-enzymatic technique (Bergmeyer, 1974), respectively.

2.5. Thin layer chromatography (TLC) analysis

Ganglioside species were analyzed by TLC and this technique was performed on 10 cm × 10 cm Merck plates of silica gel 60 using a developing tank described by Nores et al. (1994). Aliquots of the total lipid extracts containing 4 nmol of NeuAc suspended in 10 μL C:M (1:1) were spotted on 8 mm lanes. TLC was developed, sequentially, with two mixtures of solvents, firstly C:M (4:1, v/v) and secondly C:M:0.25% $CaCl_2$ (60:36:8, v/v/v). Ganglioside profile was visualized with resorcinol reagent (Svennerholm, 1957; Lake and Goodwin, 1976). The chromatographic bands were quantified by scanning densitometry at 580 nm with a CS 9301 PC SHIMADZU densitometer. Individual ganglioside values, expressed as nmol NeuAc/mg protein, were calculated by relating their respective percentage to the absolute total quantity of ganglioside-NeuAc. The terminology used herein for gangliosides is that recommended by Svennerholm (1963).

Phospholipid species were analyzed by TLC using chloroform:methanol:acetic acid:water (C:M:Aac:W, 86:14:4:1, v/v/v/v) as the solvent system which is a modification of the theoretical under phase (Folch et al., 1957). Aliquots of total lipid extracts containing a quantity equivalent to 7 μmol of inorganic phosphorus (Pi) suspended in 10 μL of C:M (2:1) were spotted on same plate size described above. Phospholipid bands were visualized with Comassie-Blue R250 (Nakamura and Handa, 1984). The chromatographic bands were quantified by scanning densitometry at 500 nm with a CS 9301 PC SHIMADZU densitometer. Individual phospholipid values, expressed as nmol Pi/mg protein, were calculated by relating their respective percentage to the absolute total quantity of phospholipid-Pi.

2.6. Preparation of synaptic plasma membrane

The cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 0.32 M sucrose solution containing 5.0 mM HEPES and 0.1 mM EDTA, pH 7.4. After homogenization, synaptic plasma membranes were prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al., 1995). These membranes were isolated using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at $69,000 \times g$ for 2 h, the fraction between 0.8 and 1.0 M sucrose interface was taken as the membrane enzyme preparation.

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

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in final volume of 200 μL. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays, as described by Wyse et al. (2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

2.8. Thiobarbituric acid-reactive substances (TBARS)

TBARS was determined according to the method described by Ohkawa et al. (1979). Briefly, 50 μL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid were added to 500 μL of cerebral cortex homogenate in a Pyrex tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifuged at $1000 \times g$ for 10 min. The organic layer was taken and the resulting pink color was determined in a spectrophotometer at 535 nm. The results were reported as nmol TBARS/mg protein.

2.9. Protein determination

Protein content was measured by the method of Bradford (1976). Bovine serum albumin was used as standard.

2.10. Statistical analysis

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

3. Results

We initially evaluated the effect of chronic hypermethioninemia on total lipid content in cerebral cortex of rats. As can be seen in Table 1, chronic administration of Met significantly decreased gangliosides [$t(13) = 5.53$; $p < 0.01$], phospholipids [$t(13) = 5.77$; $p < 0.01$] and cholesterol [$t(14) = 5.01$; $p < 0.01$] content around 60, 45 and 35%, respectively.

We also evaluated the content of the different gangliosides and phospholipids (sphingomyelin—SM, phosphatidylcholine—PC and phosphatidylethanolamine—PE) in cerebral cortex of rats subjected to chronic Met administration. Table 2 shows that chronic hypermethioninemia significantly decreased the gangliosides studied [GM1: ($t(9) = 10.65$; $p < 0.01$); GD1a: ($t(9) = 22.95$; $p < 0.01$); GD1b: ($t(9) = 7.58$; $p < 0.01$); GT1b: ($t(9) = 13.47$; $p < 0.01$)]. We also observed a reduction of all classes of phospholipids studied [SM: ($t(12) = 6.76$; $p < 0.01$); PC: ($t(12) = 13.41$; $p < 0.01$); PE: ($t(12) = 21.41$; $p < 0.01$)].

Na$^+$,K$^+$-ATPase activity and TBARS levels were also studied in cerebral cortex of rats subjected to chronic hypermethioninemia. Table 3 shows that Met-treated rats presented a significant reduction (25%) of Na$^+$,K$^+$-ATPase activity [$t(6) = 4.13$; $p < 0.01$] and an increase (30%) of TBARS values [$t(6) = -4.65$; $p < 0.01$] when compared to control groups.

Table 1

Effect of chronic hypermethioninemia on ganglioside, phospholipid and cholesterol content in cerebral cortex of rats

Lipid content	Control	Methionine
Gangliosides	265.55 ± 31.43	100.12 ± 5.95[*]
Phospholipids	786.09 ± 53.47	425.11 ± 35.24[*]
Cholesterol	123.86 ± 7.95	80.91 ± 3.18[*]

Ganglioside, phospholipid and cholesterol content are expressed in nmol NeuAc/mg protein, nmol Pi/mg protein and μg cholesterol/mg protein, respectively. Data are presented as mean ± S.E.M. for 7–8 animals in each group.

[*] Different from control, $p < 0.01$ (Student's t-test).

Table 2

Effect of chronic hypermethioninemia on ganglioside and phospholipid species in cerebral cortex of rats

	Control	Methionine
Ganglioside (nmol NeuAc/mg protein)		
GM1	33.85 ± 1.27	16.45 ± 0.99[*]
GD1a	141.40 ± 4.64	46.05 ± 1.80[*]
GD1b	27.22 ± 1.67	12.59 ± 1.11[*]
GT1b	62.81 ± 3.43	24.74 ± 0.99[*]
Phospholipid (nmol Pi/mg protein)		
SM	25.89 ± 1.43	12.75 ± 1.31[*]
PC	279.62 ± 8.20	160.32 ± 3.43[*]
PE	494.11 ± 10.77	252.03 ± 3.43[*]

Individual ganglioside and phospholipid values were calculated by relating their respective percentage to the absolute total quantity of ganglioside-NeuAc and phospholipid-Pi, respectively (see Table 1). The percentages were obtained by scanning densitometry of the TLC. Data are presented as mean ± S.E.M. for 4–7 animals in each group.

[*] Different from control, $p < 0.01$ (Student's t-test).

Table 3

Effect of chronic administration of methionine on Na$^+$,K$^+$-ATPase activity and thiobarbituric acid-reactive substances (TBARS) in cerebral cortex of rats

	Control	Methionine
Na$^+$,K$^+$-ATPase activity (nmol Pi/min·mg protein)	1091.5 ± 8.34	858.3 ± 55.76[*]
TBARS level (nmol/mg protein)	2.97 ± 0.11	3.80 ± 0.14[*]

Data are expressed as mean ± S.E.M. for 4 animals in each group.

[*] Different from control, $p < 0.01$ (Student's t-test).

4. Discussion

There is a growing body of evidence suggesting that elevated Met concentrations and/or its metabolites are potentially toxic (Benevenga and Steele, 1984; Regina et al., 1993; Mudd et al., 2001; Garlick, 2006). In this context, we have previously demonstrated that Met *in vitro* reduces brain energy metabolism, induces oxidative stress and inhibits Na$^+$,K$^+$-ATPase activity in rat hippocampus (Streck et al., 2002, 2003; Stefanello et al., 2005). Besides, we also have showed that Met administration decreases Na$^+$,K$^+$-ATPase activity and induces lipid peroxidation in hippocampus of rats (Stefanello et al., 2007b).

In the present study we initially investigated the effect of chronic administration of Met on total lipid content in cerebral cortex of rats, by using an experimental model of hypermethioninemia developed in our laboratory (Stefanello et al., 2007a). Results demonstrated that chronic hypermethioninemia significantly reduced the total content of gangliosides, phospholipids and cholesterol in cerebral cortex of rats. Therefore, considering that Met was administered in a period characterized by rapid development of central nervous system and intense cellular proliferation and growth (Loo et al., 1980; Davis and Squire, 1984; Clark et al., 1993), and that this phase is also characterized by a progressive accumulation of lipids particularly sphingolipids (Dobbing and Sands, 1971; Ando, 1983), it is conceivable that Met could alter normal brain development in rats subjected to long-term Met administration.

The profile of gangliosides after chronic administration of Met in rat cerebral cortex was also evaluated in our study. Results showed that Met significantly reduced the quantity of the major gangliosides (GM1, GD1a, GD1b and GT1b). These results could indicate that Met causes alterations in brain plasma membranes, since gangliosides are closely associated with neuronal membranes and participate in many neuronal functions (Tettamanti and Riboni, 1994; Mocchetti, 2005).

We also determined the phospholipid classes in cerebral cortex after Met administration. We observed a reduction in the content of the different phospholipids studied (SM, PC, and PE). In agreement with our study, alterations in brain phospholipid composition have been also reported in other models of brain injury, such as hypoxia/ischemia (Ramirez et al., 2003) and schizophrenia (du Bois et al., 2005).

Since Met administration alters membrane lipids composition that can alter membrane fluidity and permeability and Na^+,K^+-ATPase is considered a marker of neuronal membrane (Jones and Matus, 1974), in the present study we also investigated the effect of chronic Met administration on this enzyme activity in synaptic plasma membrane from cerebral cortex of rats. Results showed that Na^+,K^+-ATPase activity was significantly reduced by long-term Met administration. This result is in agreement with our previous in vitro and in vivo data showing that Met inhibits hippocampal Na^+,K^+-ATPase activity (Stefanello et al., 2005, 2007b).

It has been demonstrated that the oxidative membrane damage can affect the cellular dynamic properties, and consequently the function of membrane-bound enzymes, which may have serious consequences on neuronal functioning (Mecocci et al., 1997; Kamboj et al., 2006). We observed here that rats subjected to Met administration present an increase of TBARS (index of lipid peroxidation) in cerebral cortex. This result is in agreement with previous studies showing that Met increases lipid peroxidation in hippocampus of rats (Stefanello et al., 2005, 2007b). We propose that the alterations caused by Met could be, at least in part, due to oxidative damage of the membrane lipids. This fact could provoke changes in lateral assembly of glycosphingolipids, unsaturated glycerophospholipids and cholesterol and alter Na^+,K^+-ATPase activity as suggested by other investigators (Lehotsky et al., 2002; Suzuki, 2002; Molander-Melin et al., 2005; Welker et al., 2007).

In conclusion, the present study shows that chronic hypermethioninemia decreases the content of the major categories of lipids (gangliosides, cholesterol and phospholipids), reduces the activity of the Na^+,K^+-ATPase and increases lipid peroxidation in cerebral cortex of rats. If confirmed in human beings, our results in association with other studies might contribute, at least in part, to the neurophysiopathology observed in hypermethioninemic patients. However, more studies are necessary to investigate additional mechanisms involved in severe hypermethioninemia.

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CAPÍTULO V – ARTIGO 5

Hypermethioninemia increases cerebral acetylcholinesterase activity and impairs memory in rats

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Hypermethioninemia Increases Cerebral Acetylcholinesterase Activity and Impairs Memory in Rats

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Abstract In the present study we investigated the effect of chronic hypermethioninemia on rat performance in the Morris water maze task, as well as on acetylcholinesterase (AChE) activity in rat cerebral cortex. For chronic treatment, rats received subcutaneous injections of methionine (1.34–2.68 μmol/g of body weight), twice a day, from the 6th to the 28th day of age; control rats received the same volume of saline solution. Groups of rats were killed 3 h, 12 h or 30 days after the last injection of methionine to AChE assay and another group was left to recover until the 60th day of life to assess the effect of early methionine administration on reference and working spatial memory of rats. AChE activity was also determined after behavioral task. Results showed that chronic treatment with methionine did not alter reference memory when compared to saline-treated animals. In the working memory task, we observed a significant days effect with significant differences between control and methionine-treated animals. Chronic hypermethioninemia significantly increased AChE activity at 3 h, 12 h or 30 days after the last injection of methionine, as well as before or after behavioral test. The effect of acute hypermethioninemia on AChE was also evaluated. For acute treatment, 29-day-old rats received one single injection of methionine (2.68 μmol/g of body weight) or saline and were killed 1, 3 or 12 h later. Results showed that acute administration of methionine did not alter cerebral cortex AChE activity. Our findings suggest that chronic experimental hypermethioninemia caused cognitive dysfunction and an increase of AChE activity that might be related, at least in part, to the neurological problems presented by hypermethioninemic patients.

Keywords Hypermethioninemia · Metabolic disease · Memory · Morris water maze · Acetylcholinesterase

Introduction

Tissue accumulation of methionine (Met) can occur in many metabolic disorders, such as methionine adenosyltransferase deficiency and homocystinuria. Affected patients with these disorders present a variable degree of neurological dysfunction, including mental retardation, cognitive deficit and cerebral edema; however the exact mechanisms involved in these alterations remain poorly understood [1, 2].

It has been described that the impairments in learning, memory and behavior observed in patients with dementia are caused, at least in part, by changes in cholinergic system function [3–6], since there are consistent evidence that low levels of acetylcholine (ACh) in the brain are associated with cognitive dysfunction [7, 8].

Cholinergic transmission is mainly terminated by ACh hydrolysis by the enzyme acetylcholinesterase (AChE; EC 3.1.1.7). This enzyme is widely expressed in tissues that receive cholinergic innervations, such as neurons and muscle cells [9, 10]. On the other hand, reports suggest that AChE substantially contributes to synaptic transmission, both in cholinergic and other types of synapses, like dopaminergic and glutamatergic ones [11].

Considering that hypermethioninemic patients usually present a variable degree of mental retardation and other neurological symptoms, we decided to investigate whether early chronic administration of Met would affect learning/

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memory in the Morris water maze, using both reference and working memory protocols. We also investigated the effect of acute and chronic administration of Met on AChE activity in cerebral cortex of rats. The working hypothesis is that chronic experimental hypermethioninemia would cause changes in AChE activity and impairment learning/memory in adult rats.

Experimental procedure

Animals and reagents

Male Wistar rats were obtained from Central Animal House of the Department of Biochemistry, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air-conditioned constant temperature ($22 \pm 1°C$) colony room, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the official governmental guidelines in compliance with the Society Policy and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Methionine administration procedure

Methionine was dissolved in 0.9% NaCl solution and buffered to pH 7.4. As for the chronic treatment, Met solution was administered subcutaneously twice a day at 8 h intervals from the 6th to 28th day of age. Control animals received saline solution in the same volumes as those applied to Met-treated rats. Animals received 1.34 µmol Met/g body weight during the first 8 days of treatment, 2.01 µmol Met/g body weight from day 14th to 20th and 2.68 µmol Met/g body weight from day 21st to 28th. The doses of Met administered were dependent on animal age, which were determined by following pharmacokinetic parameters, apparent volume of distribution, plasmatic half-time and plasmatic clearance after Met injection [12]. Rats subjected to this treatment achieved plasma Met levels (around to 2 mmol/L) similar to those found in hypermethioninemic patients with some inherited pathological conditions [2, 13]. Maximal brain levels were achieved 60 min after Met administration (1.25 µmol/g wet tissue). About 12 h after treatment, plasma and brain Met concentrations returned to normal levels. A group of animals were killed 3 h, 12 h or 30 days after the last injection of Met and the cerebral cortex was removed to AChE determination. Another group stayed at Central Animal House of the

Department of Biochemistry until the 60th day of life to be tested in the water maze task; after that animals were killed and cerebral cortex was dissected, homogenized (1:10 w/v in 150 mM potassium phosphate buffer, pH 7.5) and centrifuged at $1000 \times g$ for 10 min. The supernatant was used for the AChE activity determination.

As for the acute treatment, 29-day-old rats received one single subcutaneous injection of Met correspondent to 2.68 µmol/g of body weight and control rats received an equivalent volume of saline [12]. The animals were killed 1, 3 or 12 h after injection and the cerebral cortex was dissected and prepared as described above.

Behavioral procedures

On the 60th day of life, animals were subjected to behavioral testing. We used the Morris water maze, an apparatus widely employed for the study of spatial learning and memory tasks [14–16].

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

Reference memory task

The task consisted of 5 training and one test session. In the acquisition phase, rats had 4 daily trials to find the platform, submerged 2 cm under the water surface, and placed on the center of one of the quadrants of the tank during all training days. For each trial, the rat was placed in water facing tank wall, in one of the 4 starting locations (N, S, W and E). The order of starting positions varied in every trial and any given sequence was not repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and, in the case of failing to find it, they were gently guided to it; all animals were allowed to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was 15–20 min [15]. One day after the last training trial, each rat was subjected to a probe trial in which the platform was removed. We measured four parameters, namely latency to cross on the location of the platform, the number of target crossings and the time spent in target (the quadrant in which the platform was located in the training sessions) and opposite quadrants. These parameters were taken as a measure for spatial memory [15].

In order to detect motor impairments that could possibly affect performance in experimental groups, the swimming

Working memory task

After 1 week, the working memory version of Morris water maze was performed. The task consisted of 4 consecutive trials per day, with a 30-s inter-trial interval, when the animals were placed in the tank facing the wall and allowed to search for the submerged platform, positioned on the center of one of the quadrants. Platform position changed every subsequent day during the four testing days. Latencies to find the platform in every first, second, third and fourth trials were calculated considering all testing days so to assess working memory performance [15].

Open field task

The task was run in a wooden box measuring $60 \times 40 \times 50$ cm with a frontal glass wall, whose floor was divided by white lines into 12 equal squares. Animals were placed facing the rear left corner of the arena and observed for 2 min. The number of squares crosses with the four paws from one square to another was indicative of motor activity [17].

Acetylcholinesterase assay

Acetylcholinesterase activity was determined according to Ellman et al. [18], with some modifications. Hydrolysis rates were measured at acetylthiocholine 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°C. About 50 µl of rat cerebral cortex supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). All samples were run in duplicate.

Protein determination

Protein was measured by the method of Bradford [19] using bovine serum albumin as standard.

Statistical analysis

Reference memory training and working memory data were analyzed by repeated measure analysis of variance (ANOVA) and data from the probe trial parameters, the open field test and AChE were analyzed by Student's *t* test or by one-way ANOVA; post hoc Duncan multiple range test was run when indicated. Descriptive statistics data were expressed as mean ± SEM. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, in a PC-compatible computer.

Results

Experiment 1: effect of hypermethioninemia on reference and working memory tasks in the Morris water maze

We observed that animals subjected to chronic hypermethioninemia did not show changes in body weight at 28-day-old (control: 69.85 ± 1.23; Met: 69.57 ± 1.25, $p > 0.05$] and 60-day-old (control: 173.07 ± 2.44; Met: 175.64 ± 4.96, $p > 0.05$); this suggests that Met administration did not cause malnutrition. That is in agreement with our previous work demonstrating that body and brain weight were not altered by Met treatment [12].

Figure 1 shows that chronic administration of Met did not affect the spatial memory acquisition phase. Both groups showed the same ability to find the platform and learn its location along the 5 days of training session. Repeated measures ANOVA (days versus groups) revealed a major days effects for both groups [$F(4,23) = 75.32$, $p < 0.01$], without any interaction between days and groups. Four parameters were evaluated in the test session,
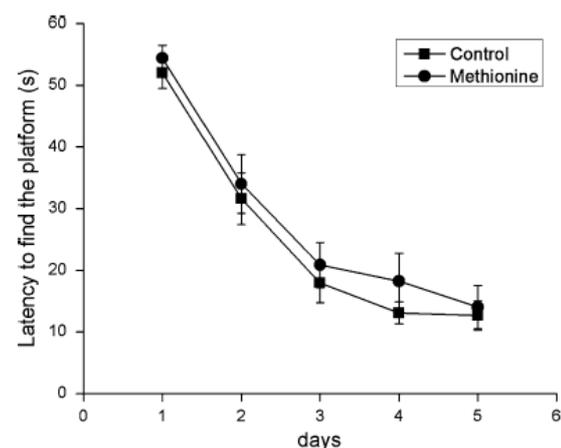
Fig. 1 Effect of chronic methionine administration on spatial memory acquisition phase. Data show latencies to find the platform across blocks of four trials on each day and are expressed as mean ± SEM for 14 animals in each group. There was no significant difference between groups, $p > 0.05$ (repeated measures ANOVA)

Table 1 Effect of chronic administration of methionine on test session parameters namely time spent in the target quadrant, time spent in the opposite quadrant, latency to cross the location of the platform, and number of crossings over platform location

Test session parameters	Group	
	Control	Methionine
Time spent in the target quadrant (s)	27.07 ± 1.90	29.14 ± 2.19
Time spent in the opposite quadrant (s)	7.79 ± 1.00	8.36 ± 1.47
Time spent to cross the platform (s)	10.93 ± 3.22	10.64 ± 2.86
Number of crossing on the platform location	5.71 ± 0.84	4.14 ± 0.44

Data are expressed as mean ± SEM for 14 animals in each group. There was no significant difference between groups, $p > 0.05$ (Student's t test)

namely the time spent in target and opposite quadrants and the latency to cross and the number of crossings on the platform location (Table 1). It was shown that Met did not affect the time spent in the target quadrant [$t(26) = -0.71$; $p > 0.05$] nor that on the opposite quadrant [$t(26) = -0.32$; $p > 0.05$], as well as the latency to cross on the location of the platform [$t(26) = 0.07$; $p > 0.05$] and the number of crossings on the former platform location [$t(26) = 1.65$; $p > 0.05$].

We also evaluated the effect of chronic Met administration in the performance of rats in the working memory version of Morris water maze. Repeated measures ANOVA revealed a significant days effect [$F(3,24) = 108.27$; $p < 0.01$] with an interaction group x days also significant [$F(3,24) = 2.795$; $p < 0.05$]. Post hoc independent t tests showed significant differences in days 1 and 4 between the two groups (both $p < 0.05$) (Fig. 2).

In order to verify whether Met treatment would affect motor activity, we submitted the animals to the open field

task. Met did not alter the number of crossings [$t(26) = 1.23$; $p > 0.05$] nor of rearings [$t(26) = -0.47$; $p > 0.05$] (Table 2). No motor deficits were also found in rats performing both water maze tasks, as assessed by swim speed; the general mean, considering all experimental groups, was 27.7 cm/s, with $p > 0.05$.

Experiment 2: effect of hypermethioninemia on AChE activity in hippocampus of rats

Considering that cholinergic system plays a crucial role in cognitive function, we verified the effect of Met administration on AChE activity in cerebral cortex of rats. Figure 3 shows that chronic hypermethioninemia significantly increased (around 30–45%) AChE activity when the rats were killed 3 h (A) [$t(7) = -2.95$; $p < 0.05$], 12 h (B) [$t(10) = -3.66$; $p < 0.01$] or 30 days (C) [$t(6) = -3.55$; $p < 0.05$] after the last injection of Met.

Additionally, we also evaluated AChE activity in cerebral cortex of rats subjected to experimental hypermethioninemia before and after behavioral tasks. As can be observed in Table 3, AChE activity were significantly increased, by approximately 50% when measured before [$t(6) = -4.43$; $p < 0.05$] and after memory testing [$t(16) = -3.43$; $p < 0.01$].

Finally, we determined the effect of acute administration of Met on AChE activity in rat cerebral cortex at 1, 3 or 12 h after injection. Results show that a single injection of Met did not alter this enzyme activity in all times tested
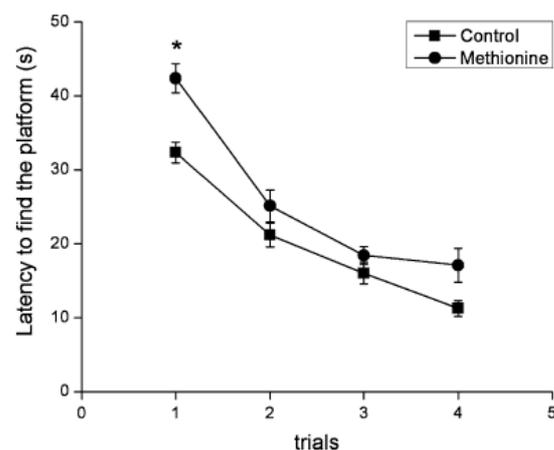
Fig. 2 Effect of chronic methionine administration on working memory version of Morris water maze. Data represent latency to find the platform on each trial during four days and are expressed as mean ± SEM for 14 animals in each group. Different from control, *$p < 0.05$ (repeated measures ANOVA)

Table 2 Effect of chronic administration of methionine on performance (number of crossings and rearings) in the open field task

Group	Number of crossings	Number of rearings
Control	37.57 ± 1.56	10.50 ± 0.87
Methionine	33.93 ± 2.51	11.14 ± 1.04

Data are presented as mean ± SEM for 14 animals in each group. There was no significant difference between groups, $p > 0.05$ (Student's t test)
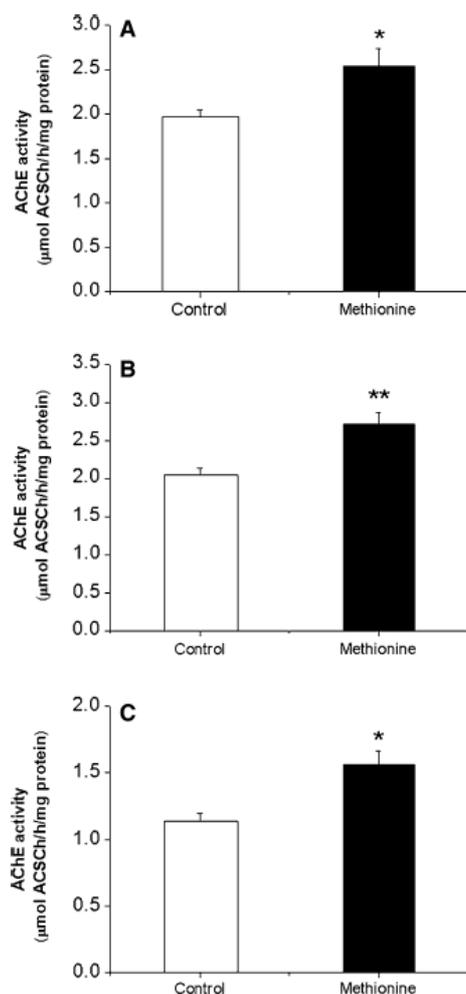
Fig. 3 Effect of chronic methionine administration on acetylcholinesterase (AChE) activity in rat cerebral cortex at 3 h (**A**), 12 h (**B**) and 30 days (**C**) after last injection. Data are expressed as mean ± SEM for 4–6 animals in each group. Different from control, *$p < 0.05$, **$p < 0.01$ (Student's t test)

[control: 1.27 ± 0.08; Met (1 h): 1.35 ± 0.02, Met (3 h): 1.17 ± 0.03, Met (12 h): 1.69 ± 0.23; $p > 0.05$].

Discussion

Neurological dysfunction is observed in patients with severe hypermethioninemia [1, 2], whose physiopathology is still poorly understood; however, there is data in literature suggesting that elevated Met concentrations and/or its metabolites are potentially neurotoxic [2, 20, 21]. We have demonstrated that Met in vitro reduces brain energy

metabolism, induces oxidative stress and inhibits Na$^+$,K$^+$-ATPase activity in rat hippocampus [22–24].

Animal models are useful to better understand the pathophysiology of diseases. In this context, we have recently developed in our laboratory a chemically experimental model of hypermethioninemia [12], in which we produced high levels of Met in blood of rats similar to those found in some pathological conditions such as methionine adenosyltransferase deficiency, homocystinuria and glycine N-methyltransferase deficiency [2, 13]. We cannot discard the increase of Met metabolites in the plasma of rats subjected to this model, such as S-adenosylmethionine, homocysteine and S-adenosylhomocysteine, perhaps cystathionine and sarcosine.

The Met was administered during a period characterized by intense synaptogenesis and gliogenesis and when cerebral structures involved in cognition have a rapid development in rats [25–28]. Animals exposed to Met treatment presented no differences in physical growth and brain weight when compared to the control group, suggesting that Met did not cause malnutrition in the rats [12].

By using this model, in the present study we investigated the effect of chronic hypermethioninemia on spatial navigation tasks in the Morris water maze. Results show that hypermethioninemic rats did not presented performance impairment in the acquisition phase (Fig. 1) nor on the time spent in target quadrant and in platform location, as well as in the latency to cross over the platform location in session (Table 1) of reference memory task. However, Met significantly impaired working memory performance, since there was significant days effect with an interaction group x days and significant differences in days 1 and 4 (Fig. 2).

We also evaluated the effect of chronic hypermethioninemia on AChE activity in cerebral cortex of rats. Our results show that this enzyme activity was increased in hypermethioninemic rats when they were killed 3 h, 12 h, 30 days after the last injection of Met (Fig. 3), as well as before and after behavioral tasks (Table 3). To confirm these findings, we verified the effect of a single injection of Met (acute study) on AChE activity. Results show that acute administration did not alter this enzyme activity in

Table 3 Effect of chronic administration of methionine on acetylcholinesterase (AChE) activity measured before and after memory tasks in rat cerebral cortex

Group	AChE activity (μmol ACSCh/h/mg protein)	
	Before	After
Control	1.13 ± 0.06	1.03 ± 0.05
Methionine	1.56 ± 0.10*	1.53 ± 0.13**

Data are expressed as mean ± SEM for 4–9 animals in each group. Different from control, *$p < 0.05$, **$p < 0.01$ (Student's t test)

cerebral cortex of rats killed 1, 3 or 12 h after Met administration, what corroborates with results of chronic treatment showing that the increase of AChE activity depend on elevated levels of Met during development. The increases in AChE may be due to the compensatory mechanism of long-term administration with Met may be due to the up-regulation of AChE gene expression. Further investigation is required to clarify the potential mechanisms by which Met and/or its metabolites act on AChE.

Although we have observed that rats subjected to chronic hypermethioninemia do not show changes on serum butyrylcholinesterase (BuChE), we cannot discard here the effect of Met on brain BuChE activity, since in our study we used a substrate that can be hydrolyzed for both cholinesterases [29]. More studies are necessary to investigate the mechanisms of increase of cholinesterases caused by chronic hypermethioninemia.

AChE, a highly conserved enzyme in animal kingdom, is distributed throughout a wide range of vertebrate tissues. It is essential to normal function of nervous system, since it rapidly terminates the action of ACh released into the synapse. The involvement of the cholinesterase in modulating glial activation, cerebral blood flux, amyloid cascade and tau phosphorylation has also been described [5, 30, 31]. It is currently speculated that actions of this enzyme could affect underlying processes in Alzheimer's disease [5], what turned AChE an important therapeutic target. In this context, reversible inhibitors of this enzyme have been used as cognitive enhancers in treatment of patients with Alzheimer's and other neurodegenerative disorders [31–35]. Furthermore, based on these data and in our results showing that chronic hypermethioninemia increases AChE activity, we could expect that the constant stimulation of this enzyme by Met might decrease ACh levels, what could be associated with memory deficits observed in hypermethioninemic rats.

In summary, the present study demonstrates that rats subjected to experimental chronic administration of Met present a significant working memory deficit and an increase of AChE activity in cerebral cortex of rats. Although it is difficult to extrapolate our results to the human condition, we cannot rule out a possible correlation between our preclinical results and the cognitive deficit found in hypermethioninemic patients.

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CAPÍTULO VI – ARTIGO 6

Hypermethioninemia provokes oxidative damage in liver of rats

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Status: A ser submetido

Hypermethioninemia provokes oxidative damage in liver of rats

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Abstract

The purpose of the present study was to evaluate the effect of early chronic administration of methionine on various parameters of oxidative stress, namely chemiluminescence, thiobarbituric acid-reactive substances (TBARS), total radical-trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), total thiol group and carbonyl content, as well as on the activities of the antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase in liver of rats. For hypermethioninemia model, Wistar rats received subcutaneous injections of methionine (1.34–2.68 µmol/g of body weight), twice a day, from the 6th to the 28th day of age and controls received saline; animals were sacrificed 3 h or 12 h after the last injection. Our data showed that chronic hypermethioninemia significantly increased chemiluminescence, carbonyl content and glutathione peroxidase activity, and decreased catalase activity when the rats were sacrificed 3 h after the last injection of methionine. In contrast, TBARS, TRAP, TAR, total thiol content and superoxide dismutase activity were not affected by methionine administration. Additionally, we also observed that hypermethioninemic rats sacrificed at 12 h after the last injection of methionine presented a significant reduction of TRAP and catalase activity, an enhance of chemiluminescence, but did not alter TAR, TBARS, total thiol group, carbonyl content, as well as the activities of glutathione peroxidase and superoxide dismutase. These findings suggest that oxidative stress induction caused by methionine may be, at least in part, one of the mechanisms related to the liver damage observed in hypermethioninemia.

Keywords: Hypermethioninemia – Oxidative stress – Liver – Rat

Introduction

It has been shown that elevation of plasma methionine (Met) may occur in several genetic abnormalities, such as methionine adenosyltransferase (MAT) activity deficiency, homocystinuria and glycine *N*-methyltransferase. Some hypermethioninemic patients can present hepatic alterations as cirrhosis and steatosis, whose underlying mechanisms are not completely established (Horowitz et al., 1981; Klatstin and Conn, 1993; Avila et al., 2000; Mudd et al., 2001; Augoustides-Savvopoulou et al., 2003).

Since the liver has an important role in the Met metabolism (Finkelstein, 1990; Mato et al., 2002), it has been proposed that elevated levels of this amino acid can be highly toxic for its (Hardwick et al., 1970; Toborek et al., 1996). In this context, some data from literature showed that disturbances of the Met cycle might result in hepatic damage, such as liver cirrhosis (Tsukamato and Lu, 2001; Avila et al., 2002, 2005).

Oxidative stress can result from increased production of reactive species that are highly reactive and can damage biomolecules, diminished levels of antioxidant or due to a combination of these conditions (Halliwell and Gutteridge, 2007). It has recently been recognized as a fundamental factor in a variety of pathophysiological changes observed in many liver diseases, such as acute hepatitis, liver cirrhosis, hepatocellular carcinoma and hepatic regeneration (Loguercio and Federico, 2003; Kim et al., 2004; Tanikawa and Torimura, 2006). In this respect, Yalçinkaya and colleagues (2007) demonstrated that Met-supplemented diet might augment hepatotoxicity and oxidative damage in liver of

ethanol-treated rats. Changes in hepatic prooxidant/antioxidant status have been reported in rats treated with high Met diet (Toborek et al., 1996; Mori and Hirayama, 2000). In addition, we have recently reported that Met *in vitro* induces oxidative stress in brain of rats (Stefanello et al., 2005).

The aim of the present study was to investigate the effect of Met on various parameters of oxidative stress in order to clarify its participation in the liver damage mechanisms observed in hypermethioninemia. To accomplish that, the effect of chronic administration of Met was studied on chemiluminescence, thiobarbituric acid-reactive substances (TBARS), total radical-antioxidant potential (TRAP), total antioxidant reactivity (TAR), total thiol group, carbonyl content, as well as on the activities of the antioxidant enzymes catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in liver of rats.

Materials and Methods

Subjects and reagents

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Science of Health, Federal University of 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 ($22 \pm 1°C$) 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 N° 80-23, revised 1996) was followed in all experiments.

All chemicals were purchased from Sigma (St. Louis, MO, USA) except 2,2'-azo-bis-(2-amidinopropane), which was purchased from Wako Chemicals (USA).

Methionine administration procedure

Methionine was dissolved in 0.9% NaCl solution and buffered to pH 7.4. For the chronic treatment, Met solution was administered subcutaneously twice a day at 8 h intervals from the 6th to 28th day of life. Control animals received saline solution in the same volumes as those applied to Met-treated rats. Met doses were calculated from pharmacokinetic parameters previously determined in our laboratory (Stefanello et al., 2007). Animals received 1.34 µmol Met/g body weight during the first 8 days of treatment, 2.01 µmol Met/g body weight from day 14th to 20th, and 2.68 µmol Met/g body weight from day 21st to 28th. Rats subjected to this treatment achieved plasma Met levels similar to those found in hypermethioninemic patients with some inherited pathological conditions (Mudd et al., 2001; Augoustides-Savvopoulou, 2003), maximal plasma levels were reached 15 min after Met injection (around to 2 mmol/L). Twelve hours after treatment, plasma Met concentrations returned to normal levels. The animals were sacrificed 3 h or 12 h after the last injection.

Tissue and homogenate preparation

Rats were sacrificed by decapitation without anesthesia, and the liver was immediately dissected out and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were

centrifuged at 750 x *g* for 10 min at 4ºC, the pellet was discarded and the supernatant was immediately separated and used for the measurements.

Chemiluminescence

Samples were assayed for chemiluminescence, a measure of lipid peroxidation, in a dark room by the method of González-Flecha et al. (1991) using a Wallac 1409 Scintillation Counter. The background chemiluminescence of incubation flasks containing 1.8 mL of the same buffer used for homogenization and 100 μL 3 mM *tert*-butyl hydroperoxide was measured for 8 min. An aliquot of 100 μL of supernatant was added to the incubation flask and incubated at 30°C for 30 min. After that, chemiluminescence was measured for 12 min at room temperature. The background chemiluminescence was subtracted from the total value. Chemiluminescence was calculated as cps/mg protein.

Thiobarbituric acid-reactive substances (TBARS)

TBARS, a measure of lipid peroxidation, was determined according to the method described by Ohkawa et al. (1979). Briefly, 50 μL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid were added to 500 μL of tissue homogenate in a Pyrex tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifuged at 1,000 x g for 10 min. The organic layer was taken and the resulting pink color was determined in a

spectrophotometer at 535 nm. The results were reported as nmol TBARS/mg protein.

Total radical-trapping antioxidant potential (TRAP)

TRAP, which represents the quantity of the tissue nonenzymatic antioxidant defenses, was determined by measuring the chemiluminescence intensity of luminol induced by 2,2'-azo-bis-(2-amidinopropane) (ABAP) thermolysis (Evelson et al., 2001) in a Wallac 1409 Scintillation Counter. The initial chemiluminescence value was obtained by adding 3 mL of 10 mM ABAP, dissolved in 50 mM sodium phosphate buffer pH 7.4, plus 10 μL of 5.6 mM luminol to a glass scintillation vial. Ten microliters of 160 μM Trolox (water-soluble α-tocopherol analogue, used as standard) or tissue supernatant were then added to that vial, producing a decrease in the chemiluminescence value until the antioxidants present are depleted and chemiluminescence reaches the initial values. The time taken by the sample to keep the chemiluminescence low is called induction time and is directly proportional to the antioxidant capacity of the tissue. The induction time of the tissue was compared to that presented by Trolox. TRAP represents the amount (quantity) of nonenzymatic antioxidants present in the sample. Results were reported as nmol Trolox/mg protein.

Total antioxidant reactivity (TAR)

TAR, which represents the quality of the tissue nonenzymatic antioxidant defenses, was determined by measuring the luminol chemiluminescence intensity

induced by ABAP thermolysis according to the method of Lissi et al. (1995) using a Wallac 1409 Scintillation Counter. The background chemiluminescence was measured by adding 4 mL of 2 mM ABAP, prepared in the same buffer used for homogenization, plus 15 µL of 4 mM luminol into a glass scintillation vial. This was considered to be the basal value. Ten microliters of 20 µM Trolox (used as a standard) or tissue supernatant was then added and the chemiluminescence was measured during 60 s to evaluate how fast it falls. The reduction in luminol intensity is considered a measure of its TAR capacity, which reflects the tissue capacity to react in front of an enhanced free radical production. TAR represents not the amount but the reactivity (quality) of nonenzymatic antioxidants present in the sample. The results were reported as nmol Trolox/mg protein.

Total thiol content

This assay was performed as described by Aksenov and Markesbery (2001), which is based on the reduction of DTNB by thiols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. Briefly, 50 µL of homogenate were added to 1 mL of PBS buffer pH 7.4 containing 1 mM EDTA. The reaction was started by the addition of 30 µL of 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). Subsequently, 30 min incubation at room temperature in a dark room was performed. Results were reported as nmol TNB/mg protein.

Protein carbonyl content

Oxidatively modified proteins present an enhancement of carbonyl content (Stadtman, 1990). Carbonyl content was assayed by the method of Reznick and Packer (1994), which is based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm. Briefly, in a dark room 100 μL of homogenate were added to plastic tubes containing 400 μL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 hour and vortexed each 15 minutes. After that, 500 μL of 20% trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 14,000 rpm for 3 minutes. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol:ethyl acetate (1:1, v/v), vortexed and centrifuged at 14,000 rpm for 3 minutes. This washing procedure was repeated once again and, after centrifugation, the supernatant was discarded and the pellet ressuspended in 600 μL of 6 M guanidine (prepared in 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60°C for 15 minutes. After that, it was centrifuged at 14,000 rpm for 3 minutes and the absorbance was measured at 370 nm (UV) in a quartz cuvette. Results were reported as nmol carbonyl/mg protein.

Catalase (CAT) assay

CAT activity was assayed according to Aebi (1984) by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100 and 10 mM potassium phosphate buffer, pH 7.0. One CAT unit

is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is reported as units/mg protein.

Glutathione peroxidase (GSH-Px) assay

GPx activity was measured according to the method described by Wendel (1981) using *tert*-butyl hydroperoxide as substrate. NADPH disappearance was monitored at 340 nm in a medium containing 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is represented as units/mg protein.

Superoxide dismutase (SOD) assay

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O_2^-, which is substrate for SOD (Marklund, 1985). The inhibition of autoxidation 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. The results were reported as units/mg protein.

Protein determination

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

Statistical analysis

Data were analyzed by unpaired Student's *t* test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p<0.05$ was considered to be significant.

Results

First, we investigated the effect of chronic Met administration on lipid peroxidation parameters in liver of rats. Figure 1A shows that chronic hypermethioninemia significantly increased chemiluminescence when measured at 3 h (up to 40%) [$t(8)=-4.19$; $p<0.01$] and 12 h (up to 25%) [$t(6)=-3.48$; $p<0.05$] after the last injection of Met. TBARS levels were not altered by this amino acid [3 h: ($t(9)=1.19$; $p>0.05$); 12 h: ($t(9)=-1.23$; $p>0.05$)] (Fig. 1B).

Next, we evaluated the effect of chronic hypermethioninemia on total antioxidant capacity of liver homogenates by determining TRAP and TAR, which evaluate nonenzymatic antioxidants quantity and reactivity, respectively. Figure 2A shows that hypermethioninemia significantly reduced TRAP when the rats were sacrificed 12 h (approximately 30%) [$t(12)=4.39$; $p<0.01$], but not 3 h [$t(8)=1.20$; $p>0.05$] after the last injection of Met. In contrast, TAR was not altered by Met at none time tested [3 h: ($t(9)=1.19$; $p>0.05$); 12 h: ($t(10)=1.58$; $p>0.05$)] (Fig. 2B).

We also investigated total thiol group and carbonyl content. As can be observed in Figure 3, total thiol content was not altered by chronic hypermethioninemia at 3 h [$t(9)=0.18$; $p>0.05$] or 12 h [$t(10)=-0.8$; $p>0.05$] after the

last injection of Met. Figure 4 shows that carbonyl content was significantly enhanced by hypermethioninemia in liver of rats when measured at 3 h (up to 50%) [$t(9)=-3.81$; $p<0.01$], but not 12 h [$t(10)=-1.57$; $p>0.05$] after the last administration of Met.

Finally, the activities of antioxidant enzymes were assayed in liver of rats subjected to chronic hypermethioninemia (Table 1). The activity of CAT was significantly decreased (approximately 30%) by Met when measured at 3 h [$t(9)=3.52$; $p<0.01$] and 12 h [$t(11)=4.63$; $p<0.01$] after last injection of this amino acid. In addition, the activity of GSH-Px was enhanced at 3 h (approximately 50%) [$t(10)=-4.83$; $p<0.01$], but not 12 h [$t(10)=0.28$; $p>0.05$] after Met administration. SOD activity was not affected by hypermethioninemia at none time tested [3 h: ($t(9)=-0.74$; $p>0.05$); 12 h: ($t(12)=0.004$; $p>0.05$)].

Discussion

In the present study we investigated the effect of chronic administration of Met on various oxidative stress parameters in rat liver, by using an experimental model of hypermethioninemia developed in our laboratory (Stefanello et al., 2007), in which we produced high levels of Met in blood of rats similar to those found in some pathological conditions such as methionine adenosyltransferase deficiency, homocystinuria and glycine N-methyltransferase deficiency (Mudd et al., 2001; Augoustides-Savvopoulou et al., 2003). According to the pharmacokinetic

parameters determined in this model, the Met concentration is relatively high at 3 h after last administration of this amino acid and returned to normal levels after 12 h.

We initially demonstrated that Met significantly increased chemiluminescence when measured at 3 h and 12 h after the last injection of the amino acid. In contrast, we did not observe alteration in TBARS levels (Fig. 1). Similar data were found in liver of rats chronically exposed to glutaric acid (Latini et al., 2007). In this scenario, it should be emphasized that the markers of lipid peroxidation utilized in our study reflect different phases of lipid oxidative damage, the light emitted in the chemiluminescence assay usually arises from peroxidizing lipids resulting from an increase in reactive species production, whereas TBARS reflects the amount of malondialdehyde formation, one important intermediates of lipid peroxidation (Halliwell and Gutteridge, 2007). Furthermore, other explanation to these findings could be the binding of malondialdehyde to biomolecules, such as structural and functional proteins, amino acids and nucleic acids (Esterbauer et al., 1991; Tuma, 2002; Aldini et al., 2007).

The next set of experiments was performed in order to evaluate whether rats sacrificed 3 h or 12 h after chronic administration of Met would be able to alter nonenzymatic antioxidant defenses in liver. We found that Met-treatment significantly decreased TRAP, but did not alter TAR at 12 h after last injection of Met. These parameters were not altered when the rats were sacrificed 3 h after last administration of amino acid (Fig. 2). Our results indicate that Met compromises the liver nonenzymatic antioxidant quantity, since TRAP measures the content of nonenzymatic antioxidant defenses, while TAR reflects the capacity of a tissue to

prevent the damage associated to reactive species processes (Lissi et al., 1995; Evelson et al., 2001).

We also evaluated the effect of Met-treatment on total thiol content in liver of rats. Results showed that this parameter was not altered by chronic hypermethioninemia at 3 h or 12 h after the last injection of Met (Fig. 3). Although we cannot establish the mechanisms by which Met alters TRAP values, it is feasible that this amino acid decreases nonenzymatic antioxidants, but not GSH content, since our results demonstrated that total thiol content was not affected by chronic hypermethioninemia. However, more studies are necessary to elucidate such mechanisms.

The measurement of protein carbonyl groups is the most frequently used biomarker of protein damage (Levine, 2002; Dalle-Donne et al., 2003). Carbonyls can arise as a result of protein glycation by sugars, by the binding of aldehydes to proteins and by the direct oxidation of amino acid side chains by reactive species (Requena et al., 2001; Levine, 2002; Dalle-Donne et al., 2003). In this study, we showed that chronic hypermethioninemia significantly increased carbonyl content at 3 h after last injection of Met, but not at 12 h (Fig. 4), suggesting an effect caused by the presence of Met and/or an indirect long-term effect of this amino acid.

As regards the antioxidant enzymes, we observed that chronic hypermethioninemia provoked a significant decrease of CAT activity when the rats were sacrificed at 3 h and 12 h after last injection of Met. In addition, the activity of GSH-Px was increased only at 3 h and SOD activity was not affected by chronic Met administration in rat liver (Table 1). In accordance with our study, alterations in

these enzyme activities were found after administration of *N*-nitrosodiethylamine, a potent carcinogenic agent, in liver of rats (Bansal et al., 2005; Subramanian et al., 2007). We cannot establish precisely the mechanisms by which Met alters the CAT and GSH-Px activities; however, it has been demonstrated that the decrease of antioxidant enzyme activities may reflect the sensitivity of these enzymes to free radical-induced inactivation (Godin et al., 1988). On the other hand, enhanced activity of antioxidant enzymes may occur in response to sustained production of reactive species (Harris, 1992). In this scenario, GSH-Px activity was increased in aging as an adaptation to an enhanced level of oxidative products (de Cavanagh et al., 2000; Mecocci et al., 2000). Thus, it is feasible that the increase in GSH-Px activity caused by Met administration could be due to the increased concentration of H_2O_2 resulting of the inhibition of CAT activity since these enzymes are responsible to metabolize H_2O_2 (Halliwell and Gutteridge, 2007).

In summary, our data show that long-term Met treatment decreases nonenzymatic antioxidant defenses, increases lipid peroxidation and protein carbonylation, as well as alters activity of antioxidant enzymes. Although further studies should be necessary to better identify the mechanisms involved, these findings suggest that Met induces oxidative stress in liver of rats, which may contribute, at least in part, to the hepatic damage observed in hypermethioninemic patients.

Acknowledgements

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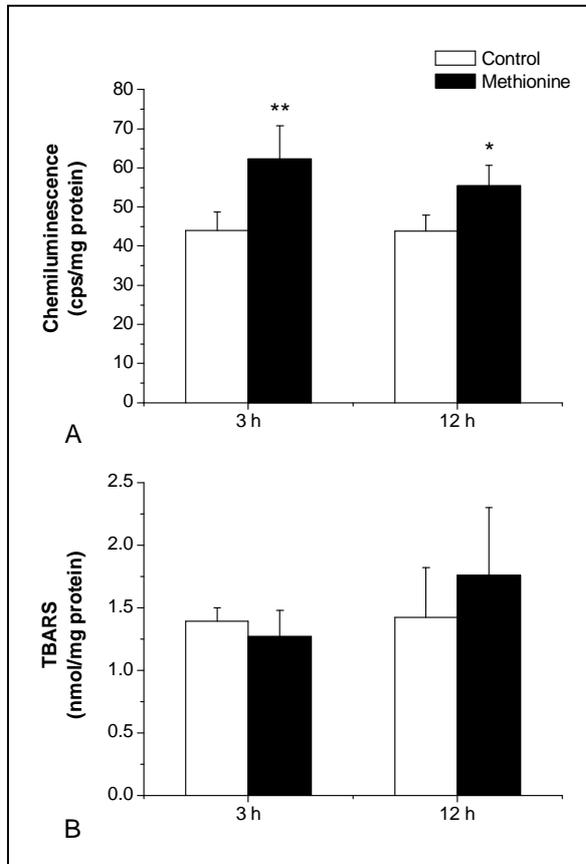
Fig. 1. Effect of chronic hypermethioninemia on chemiluminescence (A) and thiobarbituric acid-reactive substances (TBARS) (B) in rat liver at 3 h or 12 h after last administration of methionine. Data are expressed as mean $\pm$ S.D. for 4–6 animals in each group. $*p<0.05$; $**p<0.01$, compared to control (Student's t test).

Fig. 2. Effect of chronic hypermethioninemia on total radical-trapping antioxidant potential (TRAP) (A) and total antioxidant reactivity (TAR) (B) in liver of rats at 3 h or 12 h after last injection of methionine. Data are expressed as mean $\pm$ S.D. for 5–7 animals in each group. $**p<0.01$, compared to control (Student's t test).

Fig. 3. Effect of chronic administration of methionine on total thiol content in rat liver at 3 h and 12 h after last injection. Data are expressed as mean $\pm$ S.D. for 5–6 animals in each group. $p>0.05$ (Student's t test).

Fig. 4. Effect of chronic hypermethioninemia on carbonyl content in rat liver at 3 h and 12 h after last injection of methionine. Data are expressed as mean $\pm$ S.D. for 5–6 animals in each group. $**p<0.01$, compared to control (Student's t test).
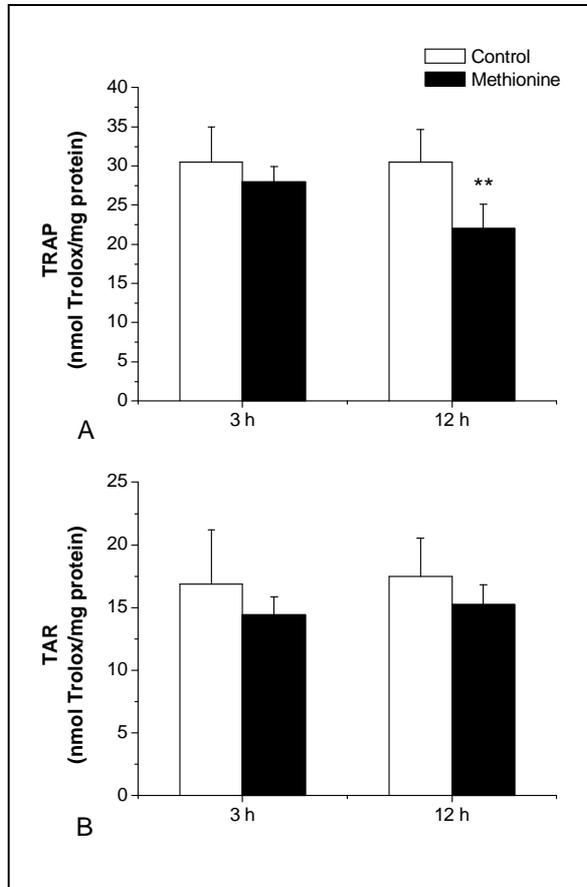
Figure 1

Figure 2

Figure 3
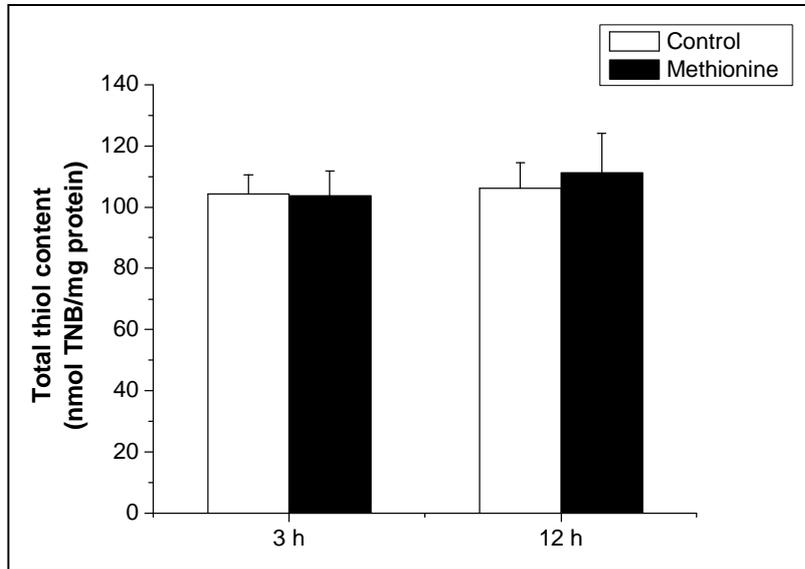
Figure 4
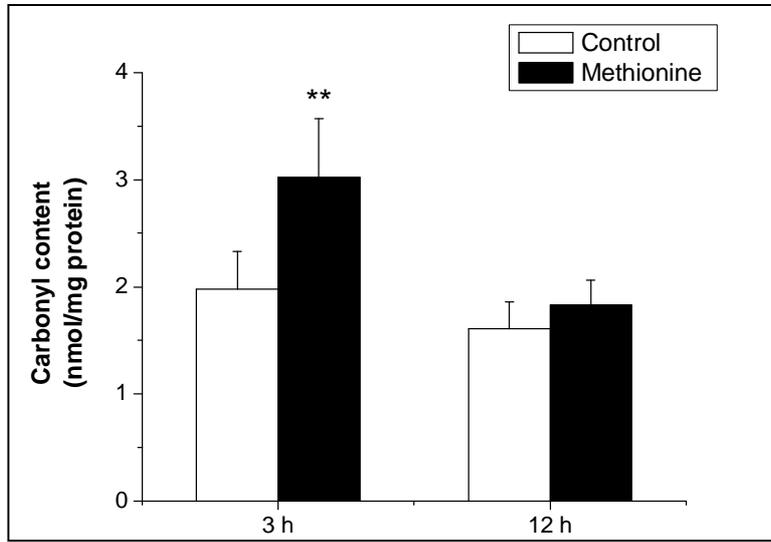
Table 1. Effect of chronic hypermethioninemia on catalase (CAT), gluthatione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities in liver of rats

Enzyme activities (units/mg protein)	Control	Methionine
3 h		
CAT	479.50 ± 88.13	329.20 ± 38.62**
GSH-Px	345.68 ± 71.81	519.54 ± 51.16**
SOD	9.98 ± 3.17	11.19 ± 1.94
12 h		
CAT	652.05 ± 105.21	465.97 ± 17.90**
GSH-Px	399.96 ± 73.98	389.47 ± 56.38
SOD	10.80 ± 1.96	10.79 ± 2.75

Results are mean ± S.D. for 5–7 animals in each group, reported as units/mg protein. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute. One GSH-Px unit is defined as 1 μmol of NADPH consumed per minute. One SOD unit is defined as 50% inhibition of pyrogallol autoxidation. **$p<0.01$, compared to control (Student's t test).

III. DISCUSSÃO

A hipermetioninemia ocorre em várias desordens metabólicas, dentre elas, na deficiência da MAT e na homocistinúria. Os pacientes afetados por essas doenças podem apresentar alterações neurológicas como déficit cognitivo, edema e desmielinização cerebral; entretanto, a patogênese dessas manifestações ainda é pouco conhecida (Mudd et al., 2000, 2001).

A neurotoxicidade da metionina tem sido evidenciada através de diversos estudos, entretanto os mecanismos pelos quais esse aminoácido atua no SNC precisam ser elucidados. Neste contexto, trabalhos realizados em nosso laboratório demonstraram que a metionina, quando adicionada diretamente ao meio de incubação (estudos *in vitro*), altera importantes parâmetros do metabolismo energético em cérebro de ratos, como a produção de CO_2, a liberação de lactato e a atividade da enzima Na^+,K^+-ATPase (Streck et al., 2002a, 2003). Mudd e colaboradores (2003) sugeriram que essas alterações causadas pela metionina poderiam explicar, pelo menos em parte, o edema cerebral presente nos pacientes hipermetioninêmicos, uma vez que a inibição da Na^+,K^+-ATPase poderia ocasionar um prejuízo no transporte dos íons Na^+ e K^+ através da membrana plasmática, com conseqüente acúmulo intracelular de Na^+ e água.

Dando continuidade aos estudos realizados por nosso grupo de pesquisa, no presente trabalho primeiramente avaliamos o efeito da pré-incubação de homogeneizados de hipocampo na presença de diferentes concentrações de metionina (0,02 a 5 mM) sobre a atividade da Na^+,K^+-ATPase em membrana plasmática sináptica de ratos. É importante salientar que utilizamos o hipocampo por ser uma estrutura essencial para os processos de memória e aprendizado (Holscher, 2003; Morris et al., 2003), e porque pacientes hipermetioninêmicos

apresentam déficit cognitivo (Mudd et al., 2000). Nossos resultados mostraram que a metionina, na concentração de 5 mM, inibe significativamente a atividade da enzima.

Alterações na atividade da Na^+,K^+-ATPase podem estar associadas a diversos mecanismos de toxicidade neuronal, uma vez que essa enzima é essencial para a manutenção do potencial de membrana necessário para a neurotransmissão (Wang et al., 2003; Yu, 2003). Neste contexto, investigadores demonstraram que uma redução na atividade Na^+,K^+-ATPase pode ocasionar morte neuronal, através da depleção de K^+ e acúmulo de Na^+ e Ca^{+2} (Xiao et al., 2002; Yu, 2003). Além disso, inibição da enzima é observada em diversas doenças neurodegenerativas (Pisani et al., 2006; Vignini et al., 2007).

Considerando que a Na^+,K^+-ATPase é inibida por espécies reativas de oxigênio (Dobrota et al., 1999; Kurella et al., 1999; Wang et al., 2003) e também por alterações na fluidez da membrana associadas à lipoperoxidação (Chakraborty et al., 2003), nós investigamos o efeito dos antioxidantes GSH (protetor de grupos −SH das proteínas) e trolox (*scavenger* de radical peroxil) sobre a inibição da Na^+,K^+-ATPase causada pela metionina em hipocampo de ratos. Os resultados mostraram que esses antioxidantes são capazes de prevenir totalmente o efeito inibitório da metionina sobre a Na^+,K^+-ATPase, sugerindo que a oxidação de grupos −SH essenciais da enzima e a peroxidação de lipídios de membrana estão envolvidos nessa inibição.

Com o propósito de verificar se a metionina induz a formação de espécies reativas, estudamos o efeito *in vitro* desse aminoácido sobre alguns parâmetros de

estresse oxidativo, denominados quimiluminescência, TBARS e TRAP, bem como sobre a atividade das enzimas antioxidantes CAT, GSH-Px e SOD em hipocampo de ratos. Nossos dados mostraram que a metionina aumenta significativamente a quimiluminescência e os níveis de TBARS, considerados marcadores de peroxidação lipídica, e diminui o TRAP, um índice de defesa antioxidante tecidual não enzimática; porém, não modifica a atividade das enzimas antioxidantes. Em conjunto, esses achados sugerem que a metionina induz estresse oxidativo, o qual é caracterizado por um desequilíbrio entre a produção de espécies reativas e as defesas antioxidantes (Halliwell & Gutteridge, 2007).

Cabe ressaltar que o estresse oxidativo é um importante evento que está relacionado com a fisiopatologia de algumas doenças neurodegenerativas, tais como as doenças de Alzheimer e Parkinson, e a esclerose amiotrófica lateral (Mancuso et al., 2006; Halliwell, 2006). Neste contexto, dano oxidativo a lipídios, proteínas e DNA, bem como redução nas defesas antioxidantes enzimáticas e não enzimáticas têm sido observadas em pacientes com essas desordens (Perry et al., 2003; Halliwell, 2006).

Sabendo-se que modelos animais são amplamente utilizados para a compreensão dos mecanismos fisiopatológicos de doenças que ocorrem em humanos e que em nosso laboratório já foram desenvolvidos modelos de vários EIM, como a fenilcetonúria (Wyse et al., 1995), a hiperprolinemia (Moreira et al., 1989) e a homocistinúria (Streck et al., 2002b), o principal objetivo desse trabalho foi desenvolver um modelo químico experimental de hipermetioninemia em ratos jovens, a fim de mimetizar, pelo menos em parte, a situação clínica observada nos pacientes hipermetioninêmicos.

Os parâmetros farmacocinéticos foram determinados após a administração aguda de metionina e mostraram que a depuração plasmática aumenta, enquanto o tempo de meia-vida diminui com o avanço da idade dos animais, sugerindo que a excreção renal e o metabolismo hepático do aminoácido não estão totalmente desenvolvidos em ratos muito jovens.

A concentração plasmática máxima de metionina (em torno de 2 mmol/L) foi atingida 15 min após uma única injeção do aminoácido, permanecendo relativamente elevada por pelo menos 6 h. Já a concentração cerebral máxima (aproximadamente 1,25 μmol/g de tecido) ocorreu 1 h após a administração e diminuiu mais lentamente do que a plasmática. Não podemos descartar que outros metabólitos, como a AdoMet, a AdoHcy, a homocisteína, a cistationina e a sarcosina estejam aumentados no plasma de ratos tratados com metionina. Porém, é improvável que o aumento desses metabólitos contribua significativamente para os efeitos observados nesse estudo.

A partir desses parâmetros, desenvolvemos um modelo crônico de hipermetioninemia, no qual as doses administradas variaram conforme o peso e a idade dos animais e foram escolhidas com o objetivo de obter níveis plasmáticos similares àqueles encontrados em pacientes hipermetioninêmicos. Os animais foram tratados do 6° ao 28° dia de vida, já que esse é um período caracterizado por intensa sinaptogênese e gliogênese, e também por um rápido desenvolvimento de estruturas cerebrais envolvidas na cognição (Loo et al., 1980; Clark et al., 1993; Miller et al., 1994). Nossos resultados mostraram que os animais submetidos ao tratamento crônico com metionina não apresentam

diferenças no peso corporal, do cérebro e do hipocampo, quando comparados aos do grupo controle, indicando que o aminoácido não causa desnutrição nos animais. Desta forma, o modelo químico desenvolvido pode ser utilizado para estudar o efeito de altas concentrações de metionina sobre parâmetros bioquímicos e comportamentais em ratos, a fim de elucidar os mecanismos fisiopatológicos da hipermetioninemia.

A seguir, utilizando o modelo descrito acima, avaliamos alguns parâmetros de estresse oxidativo, denominados TBARS, atividade da CAT e conteúdo tiólico total, bem como a atividade da Na^+,K^+-ATPase em hipocampo de ratos hipermetioninêmicos. Mostramos que a administração crônica de metionina aumenta significativamente a quantidade de TBARS e reduz a atividade da Na^+,K^+-ATPase, porém não altera a atividade da CAT e o conteúdo tiólico total. É provável que a metionina altere a atividade da Na^+,K^+-ATPase de forma indireta, pois no tratamento crônico os animais foram sacrificados 12 h após a última injeção, ou seja, quando os níveis do aminoácido já haviam retornado aos valores normais.

Considerando que a hipermetioninemia crônica altera o TBARS e a Na^+,K^+-ATPase, avaliamos também o efeito da administração aguda de metionina sobre esses parâmetros bioquímicos em hipocampo de ratos sacrificados 1 h, 3 h ou 12 h após a injeção do aminoácido. Os resultados mostraram que a metionina aumenta o TBARS e diminui a atividade da Na^+,K^+-ATPase 3 h e 12 h após a administração, mas não 1 h após. Analisando esses achados podemos sugerir que o dano oxidativo aos lipídios de membrana esteja envolvido na inibição da

Na$^+$,K$^+$-ATPase causada pela metionina, o que corrobora com os resultados obtidos *in vitro*.

A fim de dar seguimento aos estudos *in vivo*, resolvemos estudar o efeito da administração crônica de metionina sobre o conteúdo total de importantes lipídios de membrana em córtex cerebral de ratos. Vimos que a hipermetioninemia crônica reduz o conteúdo de gangliosídios, fosfolipídios e colesterol. Desta forma, considerando que a metionina foi administrada em um período de rápido desenvolvimento do SNC (Loo et al., 1980; Clark et al., 1993) e que essa fase é caracterizada pelo acúmulo progressivo de lipídios, particularmente esfingolipídios (Dobbing & Sands, 1971; Ando, 1983), é provável que o desenvolvimento cerebral esteja alterado em animais cronicamente expostos a esse aminoácido.

O perfil dos gangliosídios e fosfolipídios também foi avaliado em córtex cerebral de animais submetidos à hipermetioninemia crônica. Os resultados mostraram que a metionina diminui significativamente a quantidade dos principais gangliosídios (GM1, GD1a, GD1b e GT1b) e fosfolipídios (fosfatilcolina, fosfatidiletanolamina e esfingomielina) presentes no SNC. Corroborando com esses dados, alterações na composição cerebral desses lipídios têm sido encontradas em modelos animais de acidemias orgânicas (Trindade et al., 2002), de hipóxia/isquemia (Trindade et al., 2001; Ramirez et al., 2003) e de doenças neurodegenerativas (Farooqui et al., 2004; Barrier et al., 2007).

A manutenção da composição lipídica e das propriedades físicas das membranas é essencial para o funcionamento de enzimas e receptores associados a elas (Swapna et al., 2006). Desta forma, considerando que a Na$^+$,K$^+$-ATPase é uma proteína que está inserida na bicamada lipídica, sendo

considerada um marcador de membrana neuronal (Jones & Matus, 1974), nós avaliamos o efeito da hipermetioninemia crônica sobre a atividade dessa enzima em membrana plasmática sináptica de córtex cerebral de ratos. Verificamos que a atividade da Na^+,K^+-ATPase está diminuída em animais expostos cronicamente à metionina. Também demonstramos que esse aminoácido aumenta os níveis de TBARS em córtex cerebral de ratos. Resultados foram similares aos observados em hipocampo. Com esses achados podemos propor que a metionina causa dano oxidativo aos lipídios de membrana, o que poderia provocar mudanças na estrutura dos glicoesfingolipídios, fosfolipídios e colesterol, e conseqüentemente alterar a atividade da Na^+,K^+-ATPase, como sugerido por outros investigadores (Lehotsky et al., 2002; Suzuki, 2002; Welker et al., 2007).

Tendo em vista que pacientes hipermetioninêmicos usualmente apresentam dano neurológico e distúrbios cognitivos (Mudd et al., 2000, 2001) e que, como demonstramos nesse estudo, a hipermetioninemia crônica altera parâmetros bioquímicos importantes para os processos cognitivos, nós também avaliamos o desempenho de ratos submetidos ao modelo crônico de hipermetioninemia na tarefa do labirinto aquático de Morris, a qual permite avaliar a memória espacial de referência e de trabalho (D'Hooge & De Deyn, 2001).

As tarefas experimentais foram realizadas 31 dias após o término do tratamento crônico e consistiram de duas fases: na primeira fase, os animais foram treinados a fim de aprender a localização de uma plataforma de escape, utilizando pistas colocadas na parede da sala (mapeamento ou estratégia espacial). A seguir, os animais foram submetidos à sessão de teste para avaliação dos seguintes parâmetros: tempo gasto no quadrante alvo e no oposto ao alvo,

número de cruzamentos no local da plataforma e latência para passar pelo local onde estava a plataforma. Na etapa seguinte, realizou-se a tarefa experimental para a avaliação da memória de trabalho, na qual a posição da plataforma foi modificada a cada dia. Os resultados mostraram que os ratos submetidos à administração crônica de metionina não apresentam diferenças, em relação aos controles, na fase de aquisição da memória de referência e nos parâmetros avaliados na sessão de teste. Entretanto, a metionina prejudica significativamente o aprendizado dos animais na tarefa para avaliação da memória de trabalho. Acreditamos que o déficit à memória encontrado na hipermetioninemia seja independente das habilidades motoras dos animais, visto que não foram encontradas alterações no campo aberto e na velocidade do nado.

Considerando a importância do sistema colinérgico central para os processos cognitivos (Das et al., 2005; Ballard et al., 2005; Fodale et al., 2006), nós determinamos o efeito da hipermetioninemia sobre a atividade da AChE em córtex cerebral de ratos. Os resultados mostraram que a administração crônica de metionina aumenta a atividade da AChE em ratos sacrificados 3 h, 12 h ou 30 dias após a última injeção do aminoácido, bem como antes e após a realização das tarefas comportamentais. Entretanto, verificamos que a administração aguda de metionina não altera a atividade da enzima em nenhum dos tempos analisados (1 h, 3 h ou 12 h), o que corrobora com os dados obtidos no tratamento crônico, mostrando que o aumento na atividade da AChE depende da exposição a níveis elevados de metionina durante o desenvolvimento dos animais. Embora não saibamos o exato mecanismo responsável pelo aumento na atividade da AChE, sugerimos como hipótese a síntese aumentada da enzima ocasionada pela

administração prolongada de metionina. Adicionalmente, observamos que ratos submetidos à hipermetioninemia crônica não apresentam mudanças na atividade da BuChE sérica. Entretanto, não podemos descartar um efeito da metionina sobre a atividade dessa enzima em cérebro de ratos, uma vez que em nosso estudo utilizamos como substrato a acetilcolina, a qual é hidrolisada por ambas as colinesterases (Mesulam et al., 2002).

Convém ressaltar que a AChE, enzima essencial para o funcionamento normal do SNC, está envolvida na patogenia da doença de Alzheimer, o que a torna um importante alvo terapêutico (Ballard et al., 2005). Neste contexto, inibidores reversíveis da enzima têm sido amplamente utilizados para melhorar o déficit cognitivo presente em pacientes portadores da doença de Azheimer e de outras desordens neurodegenerativas (Giacobini, 2003; Lane et al., 2006). Desta forma, baseado nesses achados e em nossos resultados mostrando que a hipermetioninemia aumenta a atividade da AChE, podemos esperar que a constante estimulação da enzima causada pela metionina diminua os níveis de acetilcolina, o que poderia estar associado ao déficit cognitivo observado em ratos hipermetioninêmicos.

Além das alterações neurológicas presentes em pacientes hipermetioninêmicos (Mudd et al., 2000, 2001), dados da literatura mostram que a hipermetioninemia provoca alterações hepáticas (Tsukamato & Lu, 2001). Considerando esse fato e sabendo que o estresse oxidativo pode estar envolvido na patogênese de diversas doenças hepáticas (Loguercio & Federico, 2003; Tanikawa & Torimura, 2006), nós avaliamos alguns parâmetros de estresse

oxidativo em fígado de ratos sacrificados 3 h ou 12 h após a administração crônica de metionina.

Nós inicialmente demonstramos que a metionina aumenta significativamente a quimiluminescência 3 h e 12 h após a última administração do aminoácido, entretanto, não altera os níveis de TBARS. É importante destacar que os marcadores de lipoperoxidação utilizados em nosso estudo representam fases distintas do dano oxidativo aos lipídios, a luz emitida no ensaio da quimiluminescência usualmente aumenta devido aos lipídios peroxidados resultantes de um aumento na produção de espécies reativas, enquanto o TBARS reflete a quantidade de MDA formada, produto final do processo de peroxidação lipídica (Halliwell & Gutteridge, 2007). A ausência de efeito da metionina sobre o TBARS também poderia ser explicada pela ligação do MDA a biomoléculas, tais como proteínas, aminoácidos e ácidos nucléicos (Esterbauer et al., 1991; Tuma, 2002; Aldini et al., 2007).

A próxima etapa do nosso estudo foi avaliar se a hipermetioninemia crônica seria capaz de alterar a quantidade (TRAP) e/ou a reatividade (TAR) das defesas antioxidantes não enzimáticas em fígado de ratos (Lissi et al., 1995; Evelson et al., 2001). Os resultados mostraram que a metionina diminui significativamente o TRAP 12 h, mas não 3 h após a última injeção do aminoácido. O TAR não foi alterado pela administração de metionina em nenhum dos tempos avaliados. Nossos achados sugerem que a metionina compromete a quantidade de antioxidantes não enzimáticos, porém não modifica a capacidade tecidual de prevenir o dano associado à ação dos radicais livres, já que não observamos alteração nos valores do TAR. Também verificamos que o conteúdo tiólico total, o

qual reflete principalmente as concentrações de GSH, permaneceu inalterado após a administração crônica de metionina. Dessa forma, embora não possamos estabelecer os mecanismos pelos quais a metionina altera os valores do TRAP, é possível que esse aminoácido diminua outros antioxidantes não enzimáticos, uma vez que o contéudo total de grupos tióis não foi modificado nas nossas condições experimentais.

A medida do conteúdo de carbonilas é considerada o principal marcador de dano oxidativo a proteínas (Levine, 2002; Dalle-Donne et al., 2003). Sabe-se que as carbonilas podem aumentar devido à glicação protéica pelos açúcares, pela ligação de aldeídos às proteínas e pela oxidação direta das cadeias laterais dos aminoácidos causada pelos radicais livres (Requena et al., 2001; Levine, 2002; Dalle-Donne et al., 2003). Verificamos que a hipermetioninemia crônica aumenta significativamente o conteúdo de carbonilas em fígado de ratos sacrificados 3 h, mas não 12 h após a última injeção de metionina, sugerindo que a presença de metionina é indispensável para que ocorra esse efeito.

Com relação às enzimas antioxidantes, nós observamos que animais sacrificados 3 h e 12 h após o tratamento crônico com metionina apresentaram uma diminuição significativa na atividade da CAT. Em adição, a atividade da GSH-Px aumentou significativamente 3 h após a última injeção de metionina e a SOD não foi alterada em nenhum dos tempos analisados. Em concordância com esses achados, alterações na atividade dessas enzimas antioxidantes foram encontradas em fígado de ratos após a administração de *N*-nitrosodietilamina, um potente agente carcinogênico (Bansal et al., 2005; Subramanian et al., 2007). Não podemos estabelecer precisamente os mecanismos pelos quais a metionina altera

a atividade da CAT e da GSH-Px; entretanto, tem sido demonstrado que a diminuição na atividade das enzimas antioxidantes pode refletir a sensibilidade dessas à inativação induzida pelos radicais livres (Godin et al., 1988). Por outro lado, um aumento na atividade das enzimas antioxidantes pode ocorrer em resposta à produção aumentada de espécies reativas (Harris, 1992). Dessa forma, podemos propor que o aumento na atividade da GSH-Px causado pela metionina ocorra devido à concentração elevada de peróxido de hidrogênio proveniente da inibição da atividade da CAT, uma vez que essas enzimas são responsáveis pela metabolização dessa ERO (Halliwell & Gutteridge, 2007).

Em resumo, podemos sugerir que a exposição prolongada à metionina induz estresse oxidativo em fígado de ratos, uma vez que diminui as defesas antioxidantes não enzimáticas, aumenta a lipoperoxidação e a carbonilação de proteínas, e também altera a atividade das enzimas antioxidantes.

Embora pouco se conheça sobre os mecanismos responsáveis pelo dano cerebral presente nos pacientes hipermetioninêmicos, demonstramos nesse trabalho que a metionina reduz a atividade da Na^+,K^+-ATPase, induz estresse oxidativo, altera o conteúdo de importantes lipídios de membrana e aumenta a atividade da AChE em cérebro de ratos. Verificamos também que a metionina causa prejuízo à memória em ratos. Esses achados, em conjunto, podem ser relevantes para explicar as alterações neurológicas presentes na hipermetioninemia.

IV. CONCLUSÕES

ESTUDOS *IN VITRO*:

- A pré-incubação de homogeneizados de hipocampo de ratos com metionina inibiu a atividade da Na^+,K^+-ATPase de membrana plasmática sináptica. A inibição foi prevenida pelos antioxidantes GSH e trolox.

- A metionina diminuiu o TRAP e aumentou o TBARS e a quimiluminescência em hipocampo de ratos.

ESTUDOS *IN VIVO*:

- Foi desenvolvido um modelo químico experimental de hipermetioninemia em ratos, cujas concentrações plasmáticas de metionina foram semelhantes àquelas encontradas em pacientes hipermetioninêmicos (aproximadamente 2 mmol/L).

Animais submetidos ao modelo de hipermetioninemia apresentaram:

- Redução na atividade da Na^+,K^+-ATPase, aumento na atividade da AChE e nos níveis de TBARS em cérebro de ratos.

- Redução no conteúdo total de gangliosídios, fosfolipídios e colesterol. Diminuição na quantidade dos principais gangliosídios (GM1, GD1a, GD1b e GT1b) e fosfolipídios (fosfatidilcolina, fosfatidiletanolamina e esfingomielina) em cérebro de ratos.

- Prejuízo na memória de trabalho.

- Aumento na quimiluminescência, no conteúdo de carbonilas e na atividade da GSH-Px. Diminuição no TRAP e na atividade da CAT em fígado de ratos.

CONCLUSÃO GERAL

Tendo em vista que modelos animais são extremamente úteis no conhecimento da fisiopatologia de doenças que ocorrem em humanos, destacamos a importância do nosso trabalho ao desenvolver um modelo químico experimental da hipermetioninemia. Esperamos que esse modelo permita a realização de muitos estudos cuja finalidade seja melhor compreender os mecanismos envolvidos nas alterações presentes nos pacientes hipermetioninêmicos.

Em conjunto, demonstramos que a hipermetioninemia provoca uma série de alterações bioquímicas e comportamentais em ratos, as quais, se confirmadas em humanos, poderão auxiliar no entendimento da disfunção neurológica e do dano hepático associados à hipermetioninemia.

V. PERSPECTIVAS

1. Avaliar a expressão gênica da Na^+,K^+-ATPase e da AChE em hipocampo de ratos submetidos à hipermetioninemia crônica.

2. Investigar o efeito da administração crônica de metionina sobre alguns parâmetros do metabolismo energético (produção de CO_2, atividade de enzimas da cadeia respiratória) em hipocampo de ratos.

3. Investigar o efeito da administração crônica de metionina sobre outras tarefas comportamentais em ratos.

4. Investigar o efeito do tratamento com o gangliosídio GM1 sobre as possíveis alterações bioquímicas e comportamentais causadas pela hipermetioninemia crônica.

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