UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITOS DOS PRINCIPAIS ÁCIDOS ORGÂNICOS ACUMULADOS NA ACIDÚRIA 3-HIDROXI-3-METILGLUTÁRICA SOBRE A HOMEOSTASE REDOX, A RESPOSTA INFLAMATÓRIA E A FOSFORILAÇÃO DE PROTEÍNAS DO CITOESQUELETO EM CÓRTEX CEREBRAL E ESTRIADO DE RATOS JOVENS

Carolina Gonçalves Fernandes
Orientador: Prof. Dr. Moacir Wajner
Co-orientadora: Profª. Drª. Regina Pessoa Pureur

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
2015

“Uma vida não questionada não merece ser vivida.”

(Platão)

AGADECIMENTOS

À Deus, pois sem ele nada disso seria possível.

À Universidade Federal do Rio Grande do Sul e ao Departamento de Bioquímica, representado pelos professores e funcionários, por fornecerem todo o suporte necessário para o desenvolvimento desta tese.

Ao meu orientador, Professor Moacir Wajner, pela orientação e por me transmitir conhecimento, experiência e sabedoria para me tornar uma boa pesquisadora.

À minha co-orientadora, Professora Regina Pessoa Pureur, por me auxíliar com toda a sua sabedoria para realizar esta tese.

Aos meus bolsistas Clarissa, Rafael e Gilberto que foram incansáveis e muito queridos em todos esses 4 anos.

A todos os meus colegas e amigos dos laboratórios 38, 31R, 31M e 27 que estiveram sempre presentes me auxiliando direta ou indiretamente na realização desta tese.

À toda a minha família por sempre estar presente e por me apoiar em todos os meus ideais.

Aos meus pais e ao meu irmão por toda a educação, apoio, força, garra, amor, carinho e ensinamentos de vida.

Ao CNPq, pela bolsa concedida.

SUMÁRIO

PARTE I

Introdução e Objetivos

RESUMO

A deficiência da atividade da enzima 3-hidroxi-3-metilglutaril-CoA liase (HL) (OMIM 246450), também conhecida por acidúria 3-hidroxi-3-metilglutárica (HMGA), é um distúrbio genético caracterizado bioquimicamente pelo acúmulo predominante dos ácidos 3-hidroxi-3-metilglutárico (HMG), 3-metilglutárico (MGA) e 3-metilglutacônico (MGT), bem como em menor grau do ácido 3-hidroxiisovalérico em tecidos e líquidos biológicos de indivíduos afetados. Os pacientes apresentam sintomas neurológicos graves e anormalidades no córtex cerebral e gânglios da base cuja fisiopatogenia é pouco conhecida. No presente estudo investigamos os efeitos dos principais metabólitos acumulados na HMGA sobre importantes parâmetros de estresse oxidativo em estriado de ratos injetados com esses compostos e em astrócitos cultivados de córtex cerebral, bem como sobre a fosforilação de proteínas do citoesqueleto de estriado e córtex cerebral de ratos com 30 dias de idade. Assim, estudamos inicialmente os efeitos da administração intrastriatal de HMG e MGA sobre importantes parâmetros de estresse oxidativo em ratos em desenvolvimento. Nossos resultados demonstram que o HMG e o MGA induziram dano oxidativo em lipídios e proteínas. Os dois ácidos também aumentaram a oxidação da 2'-7'-diclorofluorescina (DCFH), ao passo que apenas o HMG induziu a produção de óxido nítrico. Em relação às defesas antioxidantes o HMG e o MGA provocaram uma diminuição das concentrações de glutationa reduzida e das atividades da superóxido dismutase e glutationa redutase, bem como um aumento da atividade da glutationa peroxidase. Já o HMG também causou um aumento de atividade da catalase e uma diminuição da atividade da glicose-6-fosfato desidrogenase. Finalmente observou-se que antioxidantes preveniram completamente ou atenuaram as alterações dos parâmetros de estresse oxidativo causadas pelo HMG, reforçando a participação de espécies reativas nesses efeitos. Observou-se também que MK-801, um antagonista não-competitivo do receptor glutamatérgico do tipo N-metil-D-aspartato (NMDA), preveniu alguns dos efeitos provocados pelo HMG, indicando o envolvimento do receptor NMDA no desequilíbrio redox causado por esse metabólito. Tendo em vista que os astrócitos são importantes para proteção neuronal e são susceptíveis a danos por neurotoxinas, o passo seguinte de nossa investigação foi determinar os efeitos do HMG e MGA sobre parâmetros importantes da homeostase redox e produção de citocinas em astrócitos corticais cultivados. Ambos os ácidos orgânicos reduziram a função mitocondrial astrocitária sem alterarem a viabilidade celular e também diminuíram as concentrações de glutationa reduzida. Em contrapartida, ambos metabólitos aumentaram a formação de espécies reativas (oxidação da DCFH) e também provocaram um aumento na liberação das IL-1β, IL-6 e TNFα através da via de sinalização de Erk. Finalmente investigamos os efeitos do HMG, MGA e MGT sobre a fosforilação da GFAP e subunidades NFL, NFM e NFH dos neurofilamentos (NF) em fatias de estriado e de córtex cerebral. Nossos resultados demonstraram que o HMG, o MGA e o MGT provocaram uma hipofosforilação da GFAP e todas as subunidades dos NF nas duas estruturas cerebrais. Verificamos ainda que a hipofosoforilação induzida por HMG nas proteínas do citoesqueleto foi mediada pela inibição das proteínas cinases, sem alterações de proteínas fosfatases. Assim, demonstramos que uma inibição da PKA estaria envolvida na hipofosforilação da Ser55 na região aminoterminal da NFL,

bem como uma inibição da JNK, resultaria na hipofosforilação das repetições do tipo KSP na região carboxiterminal das subunidades NFM e NFH. Observou-se também que a hipofosforilação do citoesqueleto era dependente dos receptores glutamatérgicos sinápticos e extrasinápticos (subunidade NR2B) do tipo NMDA e também do íon Ca^{2+}. Além disso, o inibidor da óxido nítrico sintase, L-NAME, e o antioxidante TROLOX (análogo hidrosolúvel da vitamina E) preveniram completamente a hipofosforilação e a inibição das atividades da PKA e da JNK causada pelo HMG. Podemos então presumir que, os presentes dados fornecem evidências sólidas de que o estresse oxidativo induzido pelo HMG e MGA em estriados de ratos e em astrócitos corticais cultivados, além de uma resposta inflamatória evidenciada nessas células neurais e uma hipofosoforilação em proteínas do citoesqueleto possam representar mecanismos patológicos importantes que contribuem, ao menos em parte, com as alterações cerebrais observadas nos pacientes afetados pela deficiência da HL.

ABSTRACT

3-Hydroxy-3-methylglutaryl-CoA lyase (HL) deficiency, also known as 3-hydroxy-3-methylglutaric aciduria (HMGA) is a genetic disorder biochemically characterized by predominant accumulation of 3-hydroxy-3-methylglutaric (HMG), 3-methylglutaric (MGA) and 3-methylglutaconic (MGT), as well as lesser amounts of 3-methylglutaconic and 3-hydroxyisovaleric acids in tissues and biological fluids of affected individuals. Clinically, the patients present neurological symptoms and basal ganglia injury, whose pathomechanisms are partially understood. In the present study, we investigated the effects of the main metabolites accumulating in HMGA on important parameters of oxidative stress in intrastriatally injected rats, in cortical cultured astrocytes, as well as on the phosphorylation of striatal and cortical cytoskeletal proteins of 30 day old rats. Thus, we first investigated the effects of the intrastiatal administration of HMG and MGA on important parameters of oxidative stress in developing rats. Our results demonstrate that HMG and MGA induced lipid and protein oxidative damage. HMG and MGA also increased 2',7'-dichlorofluorescein oxidation, whereas only HMG elicited nitric oxide production. Regarding the antioxidant defenses, both organic acids decreased reduced glutathione concentrations and the activities of superoxide dismutase and glutathione reductase and increased glutathione peroxidase activity. HMG also provoked an increase of catalase activity and a diminution of glucose-6-phosphate dehydrogenase activity. We finally observed that antioxidants fully prevented or attenuated HMG-induced alterations of the oxidative stress parameters, further indicating the participation of reactive species in these effects. We also observed that MK-801, a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor, prevented some of these effects, indicating the involvement of the NMDA receptor in the redox imbalance provoked HMG effects. Considering that astrocytes are important for neuronal protection and are susceptible to damage by neurotoxins the next step of this work was investigated the effects of HMG and MGA on important parameters of redox homeostasis and cytokine production in cortical cultured astrocytes. Both organic acids decreased astrocytic mitochondrial function withouth altering cell viability, and also decreased the concentrations of reduced glutathione. In contrast, they increased reactive species formation (DCFH oxidation) and also provoked a significant increase of IL-1β, IL-6 and TNFα release through the Erk signaling pathway. Finally, we investigated the effects of HMG, MGA and MGT on the phosphorylation of GFAP and the neurofilaments (NF) subunits NFL, NFM and NFH in striatal and cortical slices. Our results demonstrated that HMG, MGA and MGT caused hypophosphorylation of GFAP and of all NF subunits in both cerebral structures. We also found that the HMG-induced hypophosphorylation on the cytoskeletal proteins was mediated by the inhibition of protein kinases without altering protein phosphatases. Thus, we demonstrated that PKA inhibition was involved in the hypophosphorylation of Ser55 in the amino terminal region of NFL, as well as JNK inhibition resulting in the hypophosphorylation of KSPrepeats in the carboxyl terminal region of NFM and NFH subunits. It was also observed that the cytoskeletal hypophosphorylation was dependent on synaptic and extra synaptic (NR2B subunit) NMDA glutamatergic receptors and also on Ca^{2+}. Furthermore, the nitric oxide synthase inhibitor, L-NAME, and the antioxidant TROLOX (polar analog of

vitamin E) fully prevented the hypophosphorylation and the inhibition of PKA and JNK activities caused by HMG. We can then presume that all these data provide solid evidence that oxidative stress induced by HMG and MGA in rat striatum and cortical cultured astrocytes, beyond of a inflamatory response verified in this neural cell type and an hypophosphorylation of cytoskeletal proteins could represent important pathomechanisms that contribute, at least in part, the cerebral alterations observed in the patients afected by the HL deficiency.

Lista de abreviaturas

AMPc – adenosina 3',5'-monofosfato cíclico
ATP – adenosina trifosfato
CAT – catalase
CDK5 – proteína cinase dependente de ciclina 5
CREB – elemento de ligação em resposta ao AMPc
C-terminal – extremidade carboxiterminal
DCF – 2′,7′-diclorofluoresceína
DCFH – 2′,7′- dicloroflurescin
DCFH-DA – 2′,7′-diclorofluorescin diacetato
DNA – ácido desoxiribonucléico
EIM – erros inatos do metabolismo
ERN – espécies reativas de nitrogênio
ERO – espécies reativas de oxigênio
ER – espécies reativas
FI – filamentos intermediários
G6PD – glicose-6-fodfato desidrogenase
GFAP – proteína glial fibrilar ácida
GPx – glutationa peroxidase
GR – glutationa redutase
GSH – glutationa reduzida
HL – 3-hidróxi-3-metilglutaril-CoA liase
HMG – ácido -3-hidróxi-3-metilglutárico
HMGA – acidúria 3-hidróxi-3-metilglutárica
HNE – 4-hidroxinonenal
IL-1β – interleucina 1β
IL-6 – interleucina 6
JNK – cinase c-Jun N-terminal
KSP – serina-lisina-prolina
LDL – lipoproteína de baixa densidade
L-NAME – L-NG-nitroarginina metil ester
MAPK – proteínas cinases ativadas por mitógeno
MDA – malondialdeído

MF – microfilamentos

MGA – ácido 3-metilglutárico

MGT – ácido 3-metilglutacônico

MK-801 – dizocilpina

MT – microtúbulo

MTT – 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio

NF - neurofilamentos

NFH – neurofilamento de alto peso molecular

NFL – neurofilamento de baixo peso molecular

NFM – neurofilamento de médio peso molecular

NMDA – N-metil-D-aspartato

NOS – óxido nítrico sintase

NR2B – subunidade 2B do resceptor NMDA

N-terminal – extremidade aminoterminal

8-OHdGA – 8-hidroxi-2'-deoxiguanosina

OHIVA – ácido 3 hidróxiisovalérico

OMIM – online mendelian inheritance in man

PKA – proteína cinase A

PKC – proteína cinase C

PKCamII – proteína cinase dependente de cálcio e calmodulina

PP1 – proteína fosfatase 1

PP2A – proteína fosfatase 2A

PP2B – proteína fosfatase 2B ou calcineurina

PUFA – ácidos graxos de cadeia lateral polinsaturada

RNA – ácido ribonucléico

SNC – sistema nervoso central

SNP – sistema nervoso periférico

SOD – superóxido dismutase

TNFα – fator de necrose tumoral α

TROLOX – ácido 6-hidróxi-2,5,7,8-tetrametilcromano-2-carboxílico

Lista de Figuras

Lista de Tabelas

I INTRODUÇÃO

I.1 Erros Inatos do Metabolismo (EIM)

I.1.1 Conceito e classificação dos EIM

O termo erros inatos do metabolismo (EIM) foi utilizado pela primeira vez por Archibald Garrod em 1908 durante estudos realizados com pacientes com alcaptonúria, doença em que os afetados excretam grandes quantidades de ácido homogentísico na urina. O pesquisador observou que, frequentemente, um ou mais indivíduos da mesma família eram portadores da doença sem que seus pais fossem afetados. Baseado também na observação da maior incidência de consanguinidade entre os pais dos pacientes e nas leis de Mendel, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da determinação do ácido homogentísico na urina de pacientes com alcaptonúria e da observação de que esta substância era um metabólito normal da degradação da tirosina, relacionou este acúmulo a um bloqueio na conversão do ácido homogentísico até fumarato e acetoacetato. Verificou-se mais tarde que tais alterações resultavam da síntese qualitativa ou quantitativamente anormal de uma proteína enzimática (SCRIVER *et al.*, 2001).

Atualmente, mais de 500 EIM foram bioquimicamente caracterizados, sendo que a maioria envolve processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (SCRIVER *et al.*, 2001). Os pacientes portadores de algum tipo de EIM apresentam sintomatologia variada, embora haja uma predominância de sintomas neurológicos e anormalidades cerebrais. Portanto, as manifestações clínicas mais comuns nos pacientes

afetados por EIM estão relacionadas ao acometimento do SNC, com retardo neuropsicomotor, crises encefalopáticas, neurodegeneração progressiva e outras alterações neurológicas (BURTON, 1987).

A maioria dos EIM deve-se à deficiência grave na atividade de uma enzima. A ausência ou deficiência severa da atividade enzimática leva a um bloqueio da rota metabólica com acúmulo dos substratos e seus derivados que potencialmente podem provocar sintomatologia grave e mesmo morte (MITCHELL e FUKAO, 2001).

Sinclair (1982) classificou os EIM em quatro grandes grupos conforme o tipo de função exercida pela proteína deficiente e o tecido envolvido, considerando ainda aspectos clínicos, bioquímicos, patológicos e terapêuticos:

a) Distúrbios de transporte: envolvem proteínas transportadoras de moléculas orgânicas ou inorgânicas. Exemplos: deficiência de dissacaridases e defeito no transporte de magnésio.
b) Distúrbios de armazenamento, degradação e secreção: envolvem proteínas relacionadas com o aparelho de Golgi ou lisossomos na sua grande maioria, provocando acúmulo de macromoléculas em tecidos específicos. Exemplos: doenças lisossômicas de depósito, glicogenoses e cistinose;
c) Distúrbios de síntese: envolvem a síntese de proteínas com funções hormonais, de defesa imunológica, estrutural, etc. Exemplos: hiperplasia adrenal congênita por deficiência na atividade da enzima de 21-hidroxilase, hipogamaglobulinemia;
d) Distúrbios do metabolismo intermediário: caracterizam-se por

deficiências enzimáticas das rotas do metabolismo intermediário de moléculas pequenas comprometendo importantes rotas, tais como o ciclo do ácido tricarboxílico, o ciclo da uréia ou outras rotas. Assim, o substrato da enzima deficiente se acumula a menos que haja uma rota alternativa para metabolizá-lo. Por outro lado, o produto final da reação não será formado, podendo haver uma deficiência do mesmo no organismo. Os mecanismos responsáveis pelos danos teciduais podem ocorrer, desta forma pela: 1. ação do substrato acumulado que pode ser liberado na circulação e transportado para todo o organismo causando efeitos tóxicos devido a alterações bioquímicas e danos em vários tecidos e particularmente no SNC; 2. pela falta de substâncias essenciais ao desenvolvimento do organismo causada pelo bloqueio metabólico. Os mais frequentes EIM constituem os grupos das acidúrias orgânicas, as aminoacidopatias, entre outros.

A identificação de um EIM permite a introdução de um tratamento eficaz quando estiver disponível. No entanto, não há tratamento efetivo para a maioria dos EIM no momento. Além disso, a ocorrência de outros afetados no mesmo grupo familiar pode ser evitada pela identificação de casais em risco, aos quais pode ser oferecido o aconselhamento genético e, em muitos casos, o diagnóstico pré-natal (SCRIVER *et al.*, 2001).

I.1.2 Manifestações clínicas

Como mencionado anteriormente, pacientes portadores de EIM apresentam sintomas muito variados e inespecíficos, inclusive aqueles que são afetados pelo mesmo distúrbio. Essa variação fenotípica deve-se a diferentes

graus de deficiência enzimática, área do metabolismo envolvida e tecidos afetados. Algumas manifestações clínicas aparecem com maior frequência no período neonatal, tais como deficiência no crescimento, vômitos, diarréia, convulsões, letargia ou coma, odor peculiar na pele ou urina e dificuldade alimentar, enquanto outras (atraso no desenvolvimento psicomotor, neurodegeneração progressiva) podem aparecer mais tarde (BURTON, 1987). Uma gama de sintomas e sinais clínicos deve alertar o médico para esse grupo de doenças. A forma de aparecimento dos sintomas é um fator importante na distinção entre os dois grandes grupos de EIM: erros inatos devido a alterações do metabolismo intermediário e erros inatos devidos ao depósito celular de macromoléculas. Os defeitos do metabolismo intermediário que levam ao acúmulo de moléculas pequenas (ex.: aminoácidos e ácidos orgânicos) geralmente têm uma apresentação clínica súbita e a evolução se caracteriza por episódios de agudização recorrentes geralmente precedidos por infecções, ingestão alimentar exagerada de alimentos específicos, cirurgia, jejum ou outras condições de catabolismo elevado, pois nestas situações ocorre degradação de proteínas ou lipídeos que vão originar os metabólitos tóxicos (ex.: defeitos do ciclo da uréia e acidemias orgânicas). São consideradas doenças de intoxicação e/ou de comprometimento do metabolismo energético, pois várias dessas entidades patológicas caracterizam-se por prejuízo deste metabolismo.

Nos intervalos entre as crises, os pacientes podem estar clinicamente normais e a concentração dos compostos acumulados em algumas dessas doenças próxima ao normal. Para muitas destas doenças é, portanto, essencial que as amostras para análise laboratorial sejam coletadas nos momentos de

crise metabólica. O exame físico geralmente é inespecífico, assim como os exames histopatológicos dos órgãos mais afetados. O tratamento agudo com restrição alimentar específica (proteínas, lipídios ou glicídios) associada ou não à suplementação de vitaminas (que ajudam as reações enzimáticas) mostra resultados extraordinários para algumas dessas doenças, retirando o paciente da crise em poucas horas ou dias.

Outros defeitos do metabolismo intermediário mostram uma evolução crônica desde o nascimento ou nos primeiros meses ou anos de vida. Nesses casos, a intoxicação é crônica (ex.: fenilcetonúria) e os afetados apresentam um atraso na aquisição das habilidades motoras (ex.: caminhar), não adquirindo as habilidades cognitivas normais.

Já os EIM de moléculas complexas ou de organelas (doenças lisossômicas de depósito e peroxissomais) manifestam-se na sua quase totalidade de uma forma crônica e progressiva, atingindo tecidos e órgãos (fígado, baço, medula óssea e encéfalo) em que os substratos (glicogênio, lipídeos complexos, mucopolissacarídeos) que não podem ser degradados ali se depositam. É comum encontrar nesses pacientes dismorfias e sinais clínicos específicos (hepatomegalia, esplenomegalia, leucodistrofia, etc.). A doença geralmente se manifesta após os primeiros meses ou anos de vida e é comum o afetado perder as habilidades motoras e cognitivas que já tinham sido adquiridas. Os exames histopatológicos nos afetados por várias dessas doenças são bastante informativos (presença de glicogênio no fígado nas glicogenoses, de lipídeos específicos em vários tecidos nas lipidoses, etc.). O tratamento dessas doenças, no entanto, é ainda experimental para a maioria

das doenças deste grupo, sendo que a reposição enzimática tem tido recentemente sucesso em várias delas (SCRIVER *et al.*, 2001; HUGHES *et al.*, 2007; SIMON *et al.*, 2008; CHOI *et al.*, 2008; ORTIZ *et al.*, 2008; VEDDER *et al.*, 2008; GRABOWSKI, 2008; van DUSSEN *et al.*, 2014; ITIER *et al.*, 2014; ANDERSON *et al.*, 2014a,b; HENLEY *et al.*, 2014).

I.2 Acidúrias orgânicas

As acidemias ou acidúrias orgânicas constituem um grupo de EIM caracterizados pelo acúmulo de um ou mais ácidos orgânicos nos líquidos biológicos e tecidos dos pacientes afetados devido à deficiência da atividade de enzimas do metabolismo de aminoácidos, lipídeos ou carboidratos (CHALMERS e LAWSON, 1982). A frequência destas doenças na população em geral é pouco conhecida, o que pode ser creditado à falta de laboratórios especializados para o seu diagnóstico e ao desconhecimento médico sobre essas enfermidades. Na Holanda, país considerado referência para o diagnóstico de EIM, a incidência dessas doenças é estimada em 1:2.200 recém-nascidos, enquanto, na Alemanha, Israel e Inglaterra é de aproximadamente 1:6.000 - 1:9.000 recém-nascidos (HOFFMANN *et al.*, 2004). Na Arábia Saudita, onde a consanguinidade é elevada, a frequência é de 1:740 nascidos vivos (RASHED *et al.*, 1994). Chalmers e colaboradores (1980) demonstraram que as acidemias orgânicas eram os EIM mais frequentes em crianças hospitalizadas motivando diversos estudos clínicos, laboratoriais e epidemiológicos nos anos seguintes.

Clinicamente os pacientes afetados por acidemias orgânicas apresentam predominantemente disfunção neurológica em suas mais diversas formas de

expressão: regressão neurológica, convulsões, coma, ataxia, hipotonia, hipertonia, irritabilidade, tremores, movimentos coreatetóticos, tetraparesia espástica, atraso no desenvolvimento psicomotor, retardo mental e outras manifestações. As mais frequentes manifestações laboratoriais são cetose, cetonúria, neutropenia, trombocitopenia, acidose metabólica, baixos níveis de bicarbonato, hiperglicinemia, hiperamonemia, hipo / hiperglicemia, acidose lática, aumento dos níveis séricos de ácidos graxos livres e outros (SCRIVER *et al.*, 2001). Recentemente, com o uso da tomografia computadorizada e ressonância magnética, foram encontradas na maioria dos pacientes afetados por essas doenças alterações de substância branca (hipomielização e / ou desmielização), atrofia cerebral generalizada ou dos gânglios da base (necrose ou calcificação), megaencefalia, atrofia frontotemporal e atrofia cerebelar (MAYATEPEK *et al.*, 1996).

I.2.1 Acidúria 3-hidroxi-3-metilglutárica

A acidúria 3-hidroxi-3-metilglutárica (HMGA), causada pela deficiência da enzima 3-hidroxi-3-metilglutaril-CoA liase (HL; EC 4.1.3.4), é uma acidúria orgânica de herança autossômica recessiva inicialmente descrita por Faull e colaboradores (1976a,b). A HL catalisa a clivagem do 3-hidroxi-3-metilglutaril-CoA (HMG-CoA) a acetoacetato e acetil-CoA, a qual é a última reação da síntese de corpos cetônicos e do catabolismo da leucina. Estudos demonstram a existência de 90-100 casos de HMGA no mundo, sendo que, com exceção da Arábia Saudita, Península Ibérica (Portugal e Espanha) e mais recentemente no Brasil a HMGA é rara em países europeus e no Japão (VARGAS *et al.*, 2007; MENAO *et al.*, 2009).

Duas isoformas da HL são encontradas, uma localizada na mitocôndria e a outra nos peroxissomos. A HL mitocondrial é um homodímero e o gene da enzima está localizado no cromossomo 1 (1p35.1-36.1), sendo composto por 9 éxons e 8 íntrons. A isoforma mitocondrial é composta por 298 resíduos de aminoácidos e contém uma seqüência peptídica de 27 resíduos de aminoácidos na extremidade N-terminal, que sinaliza a entrada da enzima na mitocôndria. Dentro da mitocôndria, o peptídeo é removido, formando a enzima madura com 298 resíduos de aminoácidos. Por outro lado, a isoforma peroxissomal possui 325 resíduos de aminoácidos. Existe uma grande diversidade e heterogeneidade de mutações na deficiência da HL; porém, dentro de comunidades específicas o padrão pode ser mais homogêneo. As mutações c.122G>A e c.109G>A são prevalentes na Arábia Saudita e Península Ibérica, respectivamente. Apesar do conhecimento de diferentes mutações, não há correlação entre o genótipo, a atividade enzimática e o prognóstico dos pacientes (SWEETMAN e WILLIAMS, 2001; AL-SAYED *et al.*, 2006; PIÉ *et al.*, 2007).

Devido ao defeito da HL, o principal metabólito acumulado na HMGA é o ácido 3-hidroxi-3-metilglutárico (HMG), derivado da hidrólise do HMG-CoA. Na urina de pacientes em tratamento, as concentrações de HMG variam entre 200-4.000 mmol / mol de creatinina (indivíduos normais nos primeiros meses de vida: 50-90 mmol / mol de creatinina). Contudo, durante crises de descompensação metabólica, esse metabólito pode alcançar níveis de 1.500-19.000 mmol / mol de creatinina. Com a reversibilidade da reação catalisada pela enzima 3-metilglutaconil-CoA hidratase, o ácido 3-metilglutacônico (MGT) também se encontra acumulado nos tecidos e líquidos biológicos dos

pacientes. Além disso, o MGT pode ser hidrogenado a ácido 3-metilglutárico (MGA). Também devido à reversibilidade da enzima 3-metilcrotonil-CoA carboxilase, altas concentrações de 3-metilcrotonil-CoA são encontradas e este metabólito pode ainda ser hidratado, gerando ácido 3-hidroxiisovalérico (OHIVA). Além disso, durante as crises de descompensação metabólica são detectadas outros ácidos, tais como o glutárico e o adípico (BONAFÉ *et al.*, 2000; SWEETMAN e WILLIAMS, 2001).

I.2.1.1 Achados clínicos e neuropatológicos

As manifestações clínicas da HMGA se apresentam usualmente no período neonatal (SWEETMAN e WILLIAMS, 2001). Durante episódios agudos de descompensação metabólica (jejum prolongado ou situações hipercatabólicas), os pacientes apresentam vômitos, diarréia, desidratação, hipotonia, hipotermia, letargia e apnéia que pode progredir ao coma (SWEETMAN e WILLIAMS, 2001; FUNGHINI *et al.*, 2001; POSPISILOVA *et al.*, 2003). Outros sintomas incluem hepatomegalia com hiperamonemia, macrocefalia, microcefalia, pancreatite aguda, retardo no desenvolvimento e cardiomiopatia. Alguns pacientes podem evoluir até a idade adulta (LEUNG *et* al., 2009; REIMÃO *et al.*, 2009). Enfatiza-se que a deficiência da HL é fatal em aproximadamente 20 % dos casos. Os pacientes também apresentam acidose metabólica com hipoglicemia hipocetótica e elevação de transaminases no plasma. Os achados de ressonância magnética evidenciam lesões na substância branca cerebral que, em alguns casos, podem ser reversíveis com o tratamento baseado na restrição alimentar de leucina (YALÇINKAYA *et al.*, 1999; YYLMAZ *et al.*, 2006; ZAIFEIRIOU *et al.*, 2007). Além disso, foi

verificado o envolvimento dos núcleos caudato, denteado, globus pallidus, bem como do trato corticoespinhal (YALÇINKAYA *et al.*, 1999; YYLMAZ *et al.*, 2006).

I.2.1.2 Diagnóstico

A deficiência da HL deve ser investigada em pacientes que apresentam hipoglicemia hipocetótica e acidose metabólica. Geralmente a HMGA é diagnosticada através do aumento dos metabólitos acumulados HMG, MGT, MGA, 3-metilcrotonilglicina e OHIVA nos tecidos e líquidos biológicos dos pacientes (especialmente na urina) atravéz da cromatografia gasosa acoplada à espectrometria de massa. A determinação da atividade da HL também é importante, podendo ser medida espectrofotometricamente em leucócitos, fibroblastos e plaquetas. A análise de DNA também é útil para o diagnóstico pré-natal (SWEETMAN e WILLIAMS, 2001).

I.2.1.3 Tratamento

A HMGA tem tratamento disponível, baseado em dieta hipoproteica bem como a administração de L-carnitina (100 mg . kg^{-1} . dia^{-1}). Os pacientes devem evitar situações de jejum e ingesta excessiva de gordura. Além disso, a administração de glicose deve ser feita em vigência de episódios de hipoglicemia que ocorrem geralmente durante as crises. A restrição de leucina (proteína) e de gordura também é corriqueiramente utilizada (DASOUKI *et al.*, 1987).

I.2.1.4 Fisiopatologia

A patogênese da acidúria 3-hidróxi-3-metilglutárica não está bem estabelecida, no entanto, é possível que os metabólitos acumulados em tecidos e líquidos biológicos dos pacientes afetados sejam tóxicos e responsáveis por pelo menos parte dos sintomas apresentados. Os mecanismos pelos quais esses ácidos orgânicos levam aos sintomas característicos dessa doença ainda não foram totalmente desvendados, porém evidências *in vitro* e *in vivo* indicam alterações no metabolismo energético e nas oxidações biológicas.

Acredita-se que a deficiência dos corpos cetônicos (ácido acetoacético e 3-OH-butírico) durante o jejum ou durante fases agudas da doença que normalmente levam à hipoglicemia, possa explicar ao menos em parte o quadro clínico dos pacientes, já que os corpos cetônicos são uma alternativa energética importante para o cérebro.

Também foi verificado o envolvimento de espécies reativas na fisiopatologia da HMGA. Foi demonstrado que o HMG e o MGA induzem dano oxidativo lipídico e protéico e reduzem as defesas antioxidantes *in vitro* em córtex cerebral, estriado e fígado de ratos jovens (LEIPNITZ *et al*., 2008a,b; 2009). Além disso, MGA induz disfunção mitocondrial e diminui a atividade da Na^+,K^+-ATPase em sinaptossomas preparados de cérebro de ratos (RIBEIRO *et al*., 2011).

Um modelo genético nocaute de HMGA foi recentemente desenvolvido em camundongos, mas infelizmente os animais morrem ainda *in utero*,

impossibilitando, desta forma, a utilização desses animais para estudos de patogênese da doença (WANG *et al*., 1998).

I.3 Espécies reativas e radicais livres

"Espécies reativas" (ER) é um termo usado coletivamente para designar tanto espécies "radicais" quanto "não radicais", agentes oxidantes que facilmente são convertidos a radicais, tais como o ácido hipocloroso (HOCl), o ácido hipobromoso (HOBr), o ozônio (O_3), o peroxinitrito ($ONOO^-$), o oxigênio *singlet* ($^1O_2$) e o peróxido de hidrogênio (H_2O_2). Sendo assim pode-se dizer que todo radical livre é uma espécie reativa, entretanto, nem toda espécie reativa é considerada um radical livre. E dentro dessa definição enquadram-se uma ampla gama de espécies e algumas delas são listadas na Tabela I (HALLIWELL, 2006).

Radicais livres são moléculas ou átomos que contenham um ou mais elétrons desemparelhados e possuam a capacidade de existir de forma independente (SOUTHORN e POWIS, 1988; HALLIWELL e GUTTERIDGE, 1999). O desemparelhamento de elétrons, situação energeticamente instável, é o que confere alta reatividade a essas espécies. Os radicais livres podem ser formados pela perda de um elétron de um não-radical ou pelo ganho de um elétron por um não-radical. Radicais podem também ser formados em um processo de fissão homolítica, no qual uma ligação covalente é quebrada e cada elétron do par compartilhado permanece com cada um dos átomos envolvidos (HALLIWELL e GUTTERIDGE, 1999). Quando um radical livre reage com um composto não-radical, outro radical livre pode ser formado; assim, a presença de um único radical pode iniciar uma seqüência de reações

em cadeia de transferência de elétrons (redox) (MAXWELL, 1995). Nas reações em cadeia induzidas pelos radicais livres, um radical reativo leva à formação de um produto que também é um radical livre e que, por sua vez, reage produzindo um terceiro radical.

Tabela I – Algumas espécies reativas

Radicais Livres	**Não radicais**
Espécies reativas de oxigênio (ERO)	
Superóxido, $O_2^{\bullet -}$	Peróxido de hidrogênio, H_2O_2
Hidroxila, $OH^{\bullet}$	Ozônio, O_3
Hidroperoxila, $OH^{\bullet}_2$ (superóxido protonado)	*Singlet*, $^1O_2$
Carbonato, $CO^{\bullet -}_3$	Peróxidos orgânicos, ROOH
Peroxila, $RO^{\bullet}_2$	Peroxomonocarbonato, $HOOCO_2^-$
Alcoxila, $RO^{\bullet}$	Peróxicarbonato nitroso, $ONOOCO_2^-$
Radical dióxido de carbono, $CO^{\bullet -}_2$	
Espécies reativas de cloro	
Cloro atômico, $Cl^{\bullet}$	Ácido hipocloroso, HOCl
	Cloreto de nitrila, NO_2Cl
	Cloraminas
	Gás cloro, Cl_2
	Dióxido de cloro, ClO_2
Espécies reativas de bromo	
Bromo atômico, $Br^{\bullet}$	Ácido hipobromoso, HOBr
	Gás bromo, Br_2

	Cloreto de bromo, HOBr
Espécies reativas de nitrogênio (ERN)	
Óxido nítrico, $NO^{\bullet}$	Ácido nitroso, HNO_2
Dióxido de nitrogênio, $NO^{\bullet}_2$	Cátion nitrosil, NO^+
Nitrato, $NO^{\bullet}_3$	Ânion nitroxila, NO^-
	Tetróxido dinitrogenado, N_2O_4
	Trióxido nitrogenado, N_2O_3
	Peroxinitrito, $ONOO^-$
	Peroxinitrato, O_2NOO^-
	Ácido peroxinitroso, ONOOH
	Cátion nitronico, NO_2^+
	Peroxinitritos alquila, ROONO
	Peroxinitratos de alquila, RO_2ONO
	Cloreto de nitrila, NO_2Cl
	Peróxiacetil nitrato, $CH_3C(O)OONO_2$

Fonte: adaptado de Halliwell (2006)

I.3.1 Mecanismos de dano celular induzido por espécies reativas

As espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) ocorrem tanto em processos fisiológicos quanto patológicos do organismo. Fisiologicamente essas espécies reativas apresentam diversas funções (BERGENDI *et al*., 1999). Assim, um aumento da liberação local de radicais livres pode ser benéfico, como é o caso da liberação de espécies tóxicas oxidantes pelos neutrófilos, que podem atuar na defesa do hospedeiro contra uma infecção (DELANTY e DICHTER, 1998). Participam ainda de processos

de sinalização celular e também estão envolvidos na síntese e regulação de algumas proteínas (WARD e PETERS, 1995).

Por outro lado, quando formadas em excesso, essas espécies altamente reativas têm o potencial de oxidar moléculas biológicas incluindo proteínas, lipídios e DNA (MAXWELL, 1995). Com relação aos efeitos prejudiciais das reações oxidantes ao organismo, os radicais livres podem promover lipoperoxidação; podem causar a oxidação de lipoproteínas de baixa densidade (LDL); podem reagir com proteínas, levando à sua inativação e conseqüente alteração de sua função; e podem também reagir com o DNA e RNA, levando a mutações somáticas e a distúrbios de transcrição (DELANTY e DICHTER, 1998), entre outros efeitos.

I.3.1.1 Lipoperoxidação

Antes de ser vista como uma consequência de dano oxidativo, a lipoperoxidação deve ser considerada como um processo fisiológico contínuo que ocorre normalmente nas membranas celulares. Além de ser um fator de renovação da membrana, este processo é essencial na síntese de prostaglandinas e leucotrienos, bem como na fagocitose e pinocitose. No entanto, por serem formadas em grande parte por lipídios insaturados e proteínas, as membranas são particularmente vulneráveis ao ataque oxidativo. Assim, quando a produção de espécies reativas aumentar além da capacidade de detoxificação, esse processo será exacerbado, e com isso, a lipoperoxidação poderá acarretar profundas alterações na estrutura e na permeabilidade das membranas celulares. Isso irá causar perda de seletividade na troca iônica, liberação do conteúdo de organelas e formação de produtos

citotóxicos como o malondialdeído (MDA) e o 4-hidroxinonenal (HNE), entre outros eventos (FERREIRA e MATSUBARA, 1997).

Sob pH fisiológico, MDA ataca proteínas e bases do DNA, causando lesões mutagênicas (DRAPER e HADLEY, 1990). HNE pode provocar dano mitocondrial, inibição de síntese protéica e de DNA, e induz atividade quimiotáxica ou lise celular e morte (ESTERBAUER *et al.*, 1991). HNE parece ser particularmente citotóxico a células neurais (KARLHUBER *et al.*, 1997; KELLER *et al.*, 1999; MARK *et al.*, 1997). Nesse contexto, formação de HNE precede morte neuronal (ONG *et al.*, 2000) e concentrações desse produto lipídico final aumentadas são encontradas em tecido cerebral de pacientes afetados por diversas doenças neurodegenerativas (JENNER, 2003). Por lesar proteínas de membranas, esses produtos de lipoperoxidação podem também inibir a atividade de importantes enzimas de membranas, como Ca^{2+}-ATPase, glicerol-3-fosfato aciltransferase, glicose-6-fosfatase e Na^{+},K^{+}-ATPase, e também canais de potássio (DUPRAT *et al.*, 1995; McCONNELL *et al.*, 1999).

A lipoperoxidação ocorre pelas etapas de iniciação, propagação e terminação. A iniciação é causada pelo ataque a um lipídio de membrana por parte de qualquer espécie que tenha reatividade suficiente para abstrair um átomo de hidrogênio de um grupo metileno ($-CH_2-$). Nesse contexto, os radicais hidroxila podem prontamente iniciar a lipoperoxidação. Já que o átomo de hidrogênio tem apenas um elétron, a abstração de $H^{\bullet}$ de um grupo metileno deixa um elétron desemparelhado no carbono ($-{}^{\bullet}CH-$). Esse radical formado é geralmente estabilizado por um rearranjo molecular, formando um dieno conjugado. O destino mais provável desse radical é reagir com o O_2, formando

um radical peroxila ($ROO^{\bullet}$). Os radicais peroxila, por sua vez, são capazes de abstrair um próton de outra molécula lipídica, sendo esta fase conhecida como a fase de propagação. O radical de carbono formado pode reagir com o O_2 para formar outro radical peroxila, e assim sucessivamente. A abstração de um hidrogênio de outro lipídio por parte do radical peroxila gerará um hidroperóxido lipídico (LOOH). O término da reação poderá ocorrer quando dois radicais produzidos nas etapas anteriores reagirem entre si, formando um produto estável (HALLIWELL e GUTTERIDGE, 1999).

I.4 Defesas antioxidantes

Antioxidantes são substâncias endógenas ou exógenas que reduzem a formação de radicais livres ou reagem com os mesmo, neutralizando-os. A célula pode se proteger contra o dano oxidativo através de antioxidantes não enzimáticos e enzimáticos.

Embora diferindo na composição, as defesas antioxidantes estão amplamente distribuídas no organismo e compreendem agentes que removem cataliticamente os radicais livres, como as enzimas superóxido dismutase, catalase, glutationa peroxidase, entre outras; proteínas que minimizam a disponibilidade de pró-oxidantes (íons de ferro e cobre, por exemplo), ao se ligarem aos mesmos como as transferrinas; proteínas que protegem biomoléculas de danos (incluindo dano oxidativo) por outros mecanismos; agentes de baixo peso molecular que aprisionam espécies reativas de oxigênio e nitrogênio, como glutationa, α-tocoferol, ácido ascórbico e a bilirrubina.

I.4.1 Defesas antioxidantes não enzimáticas

As defesas antioxidantes não enzimáticas podem ser separadas em dois grandes grupos: agentes hidrossolúveis e lipossolúveis. O primeiro grupo inclui glutationa reduzida (GSH), ácido ascórbico, ácido úrico, melatonina, os cofatores selênio e coenzima Q10 e proteínas plasmáticas. O tripeptídeo GSH é um dos mais efetivos e abundantes antioxidantes contra ERO, particularmente no cérebro onde as concentrações alcançam 1-10mmol/L (BAST, 1993). GSH mantém o equilíbrio redox na célula e inativa ERO. Ácido ascórbico também parece ser muito importante no cérebro porque células neurais têm um sistema de captação altamente eficiente e os níveis de ácido ascórbico no líquor são muito mais altos do que no plasma (LONNROT *et al.*, 1996).

I.4.2 Defesas antioxidantes enzimáticas

A atividade de antioxidantes enzimáticos também é importante para a detoxificação de radicais livres. Exemplos desses são as izoenzimas da superóxido dismutase contendo cobre e zinco (Cu, Zn-SOD) ou magnésio (Mn-SOD), a catalase (CAT), a glutationa peroxidase (GPx) e a glutationa redutase (GR). Qualquer desequilíbrio nesse sistema pode resultar em um aumento de radicais livres derivados do oxigênio. As isoformas Cu, Zn-SOD e Mn-SOD estão presentes no citosol e mitocôndria cerebrais, e juntos com a GSH, representam a linha de defesa mais importante contra a toxicidade do $O_2^{\bullet -}$. Isoformas da SOD geram H_2O_2, o qual é removido pela atividade da GPx combinada com GSH ou GR, ou pela CAT (CHANCE *et al.*, 1979; HALLIWELL

e GUTTERIDGE, 1999b), ou adicionalmente por outras peroxidases (CHAE *et al*., 1999).

I.5 Estresse oxidativo

Espécies reativas são necessárias para a função normal da célula, servindo como moléculas de sinalização para importantes papéis fisiológicos. Elas são continuamente produzidas e neutralizadas por sistemas de defesa antioxidante. No entanto, quando produzidos em altas concentrações ou quando defesas antioxidantes estão deficientes, elas podem causar dano celular. Se o aumento de espécies reativas é relativamente pequeno, a resposta antioxidante será suficiente para compensar esse aumento. No entanto, sob certas condições patológicas, a produção de espécies reativas está muito mais aumentada, e as defesas antioxidantes podem ser insuficientes para restabelecer a homeostase redox.

O rompimento entre o equilíbrio pro-oxidante e antioxidante é descrito como estresse oxidativo, e pode representar um mecanismo fundamental de doenças humanas (HALLIWELL e GUTTERIDGE, 2007; SIES, 1985). Assim, o termo “Estresse Oxidativo” é usado para se referir à situação na qual a geração de espécies reativas ultrapassa a capacidade das defesas antioxidantes disponíveis. Pode resultar tanto de uma diminuição das defesas antioxidantes quanto de uma produção aumentada de oxidantes, bem como da liberação de metais de transição ou a combinação de quaisquer desses fatores (HALLIWELL, 2001).

O estresse oxidativo pode promover adaptação, dano ou morte celular. Quando ocorre adaptação as células podem geralmente tolerar um estresse oxidativo moderado, que geralmente resulta em *up-regulation* da síntese de sistemas de defesa antioxidante a fim de restaurar o balanço oxidante/antioxidante. Apesar disso, nem sempre o estresse oxidativo precisa envolver defesas antioxidantes aumentadas.

No dano celular, o estresse oxidativo pode danificar todos os alvos moleculares (DNA, proteínas, carboidratos e lipídios) (HALLIWELL e GUTTERIDGE, 2007). A resposta ao dano pode ser reversível: a célula entra em *steady state* alterado temporário ou prolongado que não leva à morte celular.

A morte celular pode ocorrer tanto por necrose quanto por apoptose. Na morte celular por necrose, a célula incha e se rompe, liberando seu conteúdo para o meio extracelular. Pode haver a liberação de antioxidantes, como a catalase e a glutationa, e também de pró-oxidantes, como os íons cobre e ferro e proteínas do grupo HEME, agentes esses que podem afetar as células adjacentes, podendo até mesmo impor a elas um estresse oxidativo. Já na apoptose, o mecanismo intrínseco de suicídio celular é ativado, e não há a liberação do conteúdo celular. A morte celular por apoptose pode ser acelerada em certas doenças, como as doenças neurodegenerativas, havendo envolvimento do estresse oxidativo (HALLIWELL e GUTTERIDGE, 2007).

Além da indução de necrose e apoptose, o estresse oxidativo pode levar a um aumento da lipoperoxidação, cujos produtos (MDA e HNE, entre outros) são altamente neurotóxicos, e a um dano oxidativo tanto às proteínas, inibindo

a atividade de diversas enzimas e alterando a função celular, quanto ao DNA, causando alteração de bases púricas e pirimídicas (HALLIWELL e GUTTERIDGE, 2007).

O cérebro é altamente dependente de energia para o seu funcionamento normal e a mitocôndria é a estrutura intracelular responsável pela produção dessa energia. Para a produção eficiente de energia na forma de ATP, a mitocôndria possui uma alta demanda por oxigênio, já que utiliza uma grande quantidade de O_2 em uma massa de tecido relativamente pequena, o que torna esse tecido altamente susceptível à ação de espécies reativas.

Além disso, a presença de membranas celulares ricas em ácidos graxos de cadeia lateral poliinsaturada (PUFA), os quais são especialmente sensíveis ao ataque de radicais livres e conseqüente oxidação de lipídeos pode levar ao dano por radicais livres no tecido cerebral. Alto conteúdo de ferro, o qual favorece a lipoperoxidação e autooxidação de neurotransmissores através da $OH^{\bullet}$ formado na reação de Fenton (ZALESKA e FLOYD, 1985), a considerável quantidade de microglia, macrófagos residentes do sistema nervoso que podem produzir $O_2^{\bullet -}$ e H_2O_2, sua modesta defesa antioxidante, sendo os níveis de catalase particularmente baixos em muitas regiões cerebrais (HALLIWELL e GUTTERIDGE, 1996; HALLIWELL e GUTTERIDGE, 2007), aumentam a suscetibilidade do cérebro ao dano causado por radicais livres.

Além disso, uma disfunção mitocondrial pode ocorrer por diminuição da atividade dos complexos da cadeia respiratória com um conseqüente prejuízo no transporte de elétrons, o que leva a uma dispersão dos elétrons na forma de radicais livres potencialmente danosos à célula.

Numerosas evidências sugerem que os radicais livres e o estresse oxidativo possam estar envolvidos na patogênese dos danos neurológicos de várias doenças neurodegenerativas, como doença de Alzheimer, doença de Parkinson e esclerose lateral amiotrófica. Como medida de parâmetros de estresse oxidativo, existe um considerável número de casos onde se relatou aumento nos níveis de MDA e HNE no cérebro de pacientes, além de produtos da oxidação protéica, como grupos carbonil e 3-nitrotirosina, e também produtos que resultam da oxidação de DNA, bem como concentrações reduzidas dos antioxidantes não enzimáticos GSH e ácido ascórbico e diminuição da atividade das enzimas antioxidantes CAT e GPx (JENNER e OLANOW 1996; LIU *et al.*, 1999; PERRY *et al.*, 2003).

Estudos demonstraram uma diminuição na atividade do complexo I da cadeia respiratória em cérebros *postmortem* de pacientes portadores de doença de Parkinson. Essa inibição do complexo I pode acarretar na geração de espécies reativas, tais como ânion superóxido, radicais hidroxila e peroxinitrito, as quais poderiam causar um prejuízo ainda maior na cadeia transportadora de elétrons. Dessa forma, é possível que o estresse oxidativo e as disfunções mitocondriais formem um ciclo vicioso na doença de Parkinson (GU *et al.*, 1996; JANETSKY *et al.*, 1994; SCHAPIRA *et al.*, 1989, 1990 a, b).

Na doença de Alzheimer, a mais comum dentre as doenças neurodegenerativas, é possível que o estresse oxidativo tenha um papel chave na morte neuronal. Tem sido proposto que o peptídeo β-amilóide, o formador das chamadas placas senis, tenha a capacidade de gerar radicais livres espontaneamente. Estudos *in vivo* também evidenciaram um dano oxidativo

em cérebros humanos *postmortem* com doença de Alzheimer através da observação de aumento de 8-hidroxi-2'-deoxiguanosina (8-OHdGA), produtos de oxidação de outras bases e de RNA, carbonilas de proteínas, nitrotirosina e marcadores de peroxidação lipídica (SMITH *et al.*, 1991; MARKESBERY *et al.*, 1999; NOUROOZ-ZADEH *et al.*, 1999; LOVELL *et al.*, 2000).

I.6 Citoesqueleto

Para que as células funcionem adequadamente elas devem se organizar no espaço, interagir mecanicamente com o ambiente ao seu redor, apresentar uma conformação correta, ser fisicamente robustas e estar estruturadas de forma adequada internamente. Muitas células devem também ser capazes de modificar sua forma e migrar para outros locais. Além disso, todas as células devem ser capazes de reorganizar seus componentes internos como decorrência dos processos de crescimento, divisão e/ou adaptação à mudança no ambiente. Todas essas funções estruturais e mecânicas apresentam-se muito desenvolvidas em células eucarióticas, sendo dependentes de um sistema de filamentos denominado citoesqueleto (ALBERTS *et al.*, 2008).

As diferentes atividades do citoesqueleto dependem de três tipos de filamentos protéicos: filamentos de actina ou microfilamentos (MF), microtúbulos (MT) e filamentos intermediários (FI). Cada tipo é formado pela polimerização de monômeros específicos (CARRAWAY, 2000; ALBERTS *et al.*, 2008). Os três tipos de filamentos do citoesqueleto são conectados entre si e suas funções são coordenadas, permitindo a participação em inúmeras atividades celulares em conjunto com diversas proteínas associadas (BEAR *et al.*, 2002; ALBERTS *et al.*, 2008) (Figura 1).

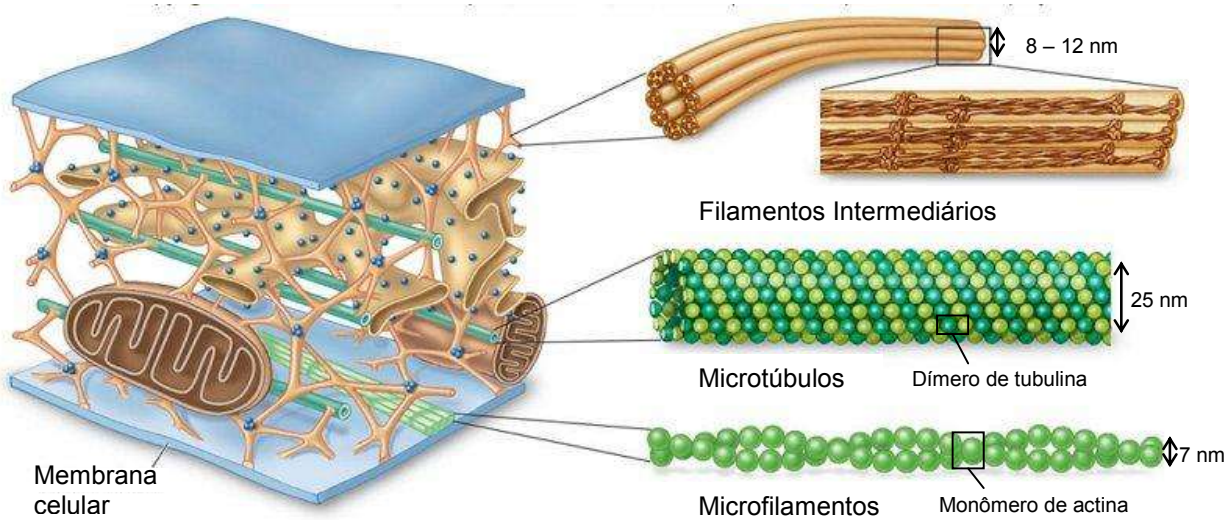


Figura 1 – Citoesqueleto das células eucarióticas. O citoesqueleto é constituído por microtúbulos, filamentos intermediários e microfilamentos. Os microfilamentos são formados por uma dupla fita e são responsáveis pela contração muscular, forma da célula e movimentos citoplasmáticos. Os filamentos intermediários são formados por subunidades que formam polímeros e ajudam na manutenção da forma celular, suportam a extensão das células nervosas e mantém as células unidas. Os microtúbulos são formados por dímeros de α e β-tubulina e estão associados com o transporte de organelas e cromossomos (adaptado de <http://www.gceadvancelevel.com/cytoskeleton/>).

I.6.1 Filamentos intermediários

Os filamentos intermediários são formados pela associação de subunidades fibrosas, constituindo fibras resistentes encontradas na maioria das células animais. Eles foram denominados intermediários por causa do seu diâmetro (8 – 10 nm), que está entre o diâmetro dos MF (7 – 8 nm) e o diâmetro dos MT (25 nm) (ALBERTS *et al.*, 2008). Os FI contituem a família mais diversificada de proteínas do citoesqueleto e são codificadas por cerca de 70 genes no genoma humano (FUCHS, 1994), possuindo um alto grau de especificidade celular e sendo frequentemente considerados marcadores de diferenciação celular (ALBERTS *et al.*, 2008, ERIKSSON *et al.*, 2009). As subunidades dos FI são constituídos por um domínio central altamente conservado em α-hélice e domínios N- e C-terminal variáveis sendo

classificados em seis sub-grupos, de acordo com o tipo celular e o padrão de desenvolvimento (FUCHS, 1994).

Inicialmente os FI eram considerados estruturas bastante estáticas e rígidas com funções relacionadas à integridade estrutural de células e tecidos (CHOU *et al*., 1997; COULOMBE *et al*., 2000; FUCHS e CLEVELAND, 1998; OMARY e KU, 1997). De fato, o papel dos FI em conferir resistência mecânica é muito relevante e a consequente perda de função pode estar relacionada com doenças envolvendo a fragilidade celular (FUCHS e CLEVELAND, 1998; OMARY *et al*., 2004). Entretanto, nos últimos anos as funções atribuídas aos FI tem ampliado significativamente, pois tem sido relacionados a uma variedade de eventos celulares. Os FI desempenham um papel ativo no transporte de sinais da periferia celular para o núcleo (CHANG e GOLDMAN, 2004; PARAMIO e JORCANO, 2002), estão envolvidos na motilidade (ECKES *et al*., 1998; HELFAND *et al*., 2003), na adesão e migração celular (IVASKA *et al*., 2005) e na modificação de vários processos celulares devido a sua habilidade de regular moléculas de sinalização (PALLARI e ERIKSSON, 2006). Outras funções atribuídas aos FI são a capacidade de agir como proteínas adaptadoras em vias de sinalização, bem como estarem envolvidos no crescimento e regeneração das células (KIM e COULOMBE, 2007) (Figura 2). Além disso, diversos trabalhos descrevem alterações dos FI em resposta a danos e nos processos de reparo tecidual, câncer e outras doenças (DePIANTO e COULOMBE, 2004; KUCHMA *el al*., 2012).

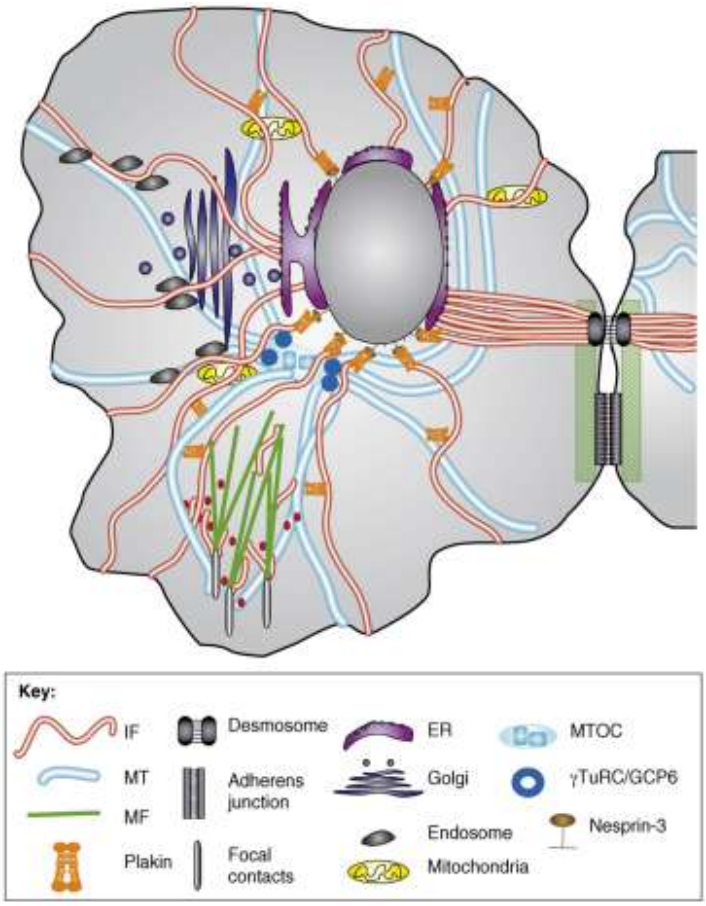


Figura 2 – Os FI são integradores da citoarquitetura. Eles estão ancorados no núcleo e se estendem para o citoplasma formando pontos de contato na membrana plasmática associando-se com a mitocôndria, às membranas do complexo de Golgi e ao sistema endolisossomal. Os FI posicionam os centros de organização dos microtúbulos (MTOC), através de interações com o complexo γTuRC/GCP6, influenciando a organização dos MT e as propriedades de suas proteínas transportadoras, bem como, da distribuição das proteínas de membrana (adaptado de Godsel e colaboradores, 2007).

No sistema nervoso central (SNC) os FI tem papel fundamental na criação e manutenção da forma de neurônios e astrócitos, na manutenção do calibre axonal, bem como no transporte de organelas e substâncias envolvidas na transmissão sináptica (KIRKPATRICK e BRADY, 1999; ACKERLEY *et al.*, 2000). Essa rede protéica é um importante alvo para mecanismos de transmissão de sinais a partir de receptores de membrana plasmática, levando a uma resposta celular apropriada. Além disso, está envolvida na organização

e reorganização dos receptores de membrana, sendo essencial para os mecanismos de reconhecimento celular (CARRAWAY, 2000), bem como para a modulação da viabilidade celular através do processo de apoptose (NDOZANGUE-TOURIGUINE *et al.*, 2008).

I.6.1.1 Neurofilamentos

Os neurofilamentos (NF) são FI importantes na fisiologia neuronal, sendo os principais componentes do citoesqueleto dos neurônios maduros. São formados pela polimerização de três subunidades: os NF de baixo (NFL; 68 KDa), médio (NFM; 150 KDa) e alto peso molecular (NFH; 200 KDa) (ALBERTS *et al.*, 2008). A extremidade amino-terminal juntamente com a região em α-hélice da subunidade NFL interagem lateralmente e longitudinalmente formando a estrutura propriamente dita do NF (GEISLER e WEBER, 1981), enquanto as regiões carboxi-terminais das subunidades NFM e NFH são responsáveis pelas projeções laterais, que permitem a interação dos NFs entre si e com os demais constituintes do citoesqueleto (HISANAGA e HIROKAWA, 1988; GOTOW *et al.*, 1992; KIRKPATRICK e BRADY, 1999). Originalmente se assumia que os NF eram compostos apenas pelas subunidades NFL, NFM e NFH, porém estudos recentes indicam que outras proteínas como a α-internexina, no SNC, e a periferina, no sistema nervoso periférico (SNP), também se associam com os NF participando da formação do FI neuronal (BEAULIEU *et al.*, 1999; YUAN *et al.*, 2006).

A principal função atribuída aos NF é a manutenção do calibre axonal e consequentemente o aumento da velocidade de condução do impulso elétrico, processo esse regulado por fosforilação. Além disso, os NF também

contribuem para as propriedades dinâmicas do citoesqueleto axonal durante a diferenciação neuronal e para o crescimento, a regeneração e a orientação dos axônios (NIXON e SHEA, 1992). Eles são formados dentro do corpo celular e posteriormente transportados para o axônio, sendo a fosforilação do domínio carboxi-terminal um importante mecanismo regulatório do transporte axonal (JUNG *et al*., 2000).

Acúmulo anormal de NF é descrito em várias doenças neurodegenerativas, tais como esclerose lateral amiotrófica, doença de Parkinson, doença de Alzheimer (AL-CHALABI e MILLER, 2003; LIU *et al*., 2004) e em neuropatias tóxicas (ZHU *et al*., 1998) sendo considerado, em muitos casos, um marcador nessas disfunções neuronais (SU *et al*., 2012). Ainda não é compreendido como esse acúmulo de NF contribui para o processo neurodegenerativo nessas doenças, mas sugere-se que o transporte dos NF esteja interrompido nos neurônios afetados (ACKERLEY *et al*., 2000; LARIVIERE e JULIEN, 2004).

I.6.1.2 Proteína glial fibrilar ácida

A proteína gial fibrilar ácida (GFAP) é uma proteína estrutural de 50 KDa sintetizada nos astrócitos e em algumas células de Schwann (GUO-ROSS *et al*., 1999; KANEKO *et al*., 1995; KOSAKO *et al*., 1997). A GFAP é considerada marcador de astrócitos e é importante na modulação da motilidade e forma celular por fornecer estabilidade estrutural aos astrócitos. Além disso, esse FI é importante para a interação astrócito-neurônio (McCALL *et al*., 1996; ELIASSON *el al*., 1999) participando do sofisticado sistema de comunicação

intercelular recíproco que pode regular a liberação de neurotransmissores, a excitabilidade neuronal e a transmissão sináptica (CARMIGNOTO, 2000).

No SNC, após um dano, os astrócitos tornam-se reativos e respondem de uma maneira típica denominada astrogliose, que é caracterizada por uma rápida síntese de GFAP (EDDLESTON e MUCKE, 1993), sendo essa proteína, em muitos casos, um marcador de neurotoxicidade. Mudanças na expressão de GFAP podem alterar a morfologia dos astrócitos o qual pode afetar indiretamente outros tipos celulares e a estrutura do cérebro (MIDDELDORP e HOL, 2011). Uma intensiva gliose reativa é verificada em doenças como esclerose múltipla (HALLPIKE *et al*., 1983), adrenoleucodistrofia (SCHAUMBURG *et al*., 1975) e doença de Alexander (ENG *et al*., 1998).

I.6.1.3 Vimentina

Entre as proteínas dos filamentos intermediários a vimentina é a mais amplamente distribuída ocorrendo em muitas células de origem mesenquimal. Além disso, é expressa de forma transitória durante o desenvolvimento em muitos tecidos (ALBERTS *et al*., 2008). No SNC é expressa na glia radial, astrócitos imaturos e também na glia de Bergmann no cerebelo. Durante a diferenciação dos astrócitos é substituída progressivamente por GFAP (MENET *et al*., 2001). Essa proteína, juntamente com a GFAP, participa do processo de gliose reativa após dano no SNC (MENET *et al*., 2001; PEKNY *et al*., 1999).

A vimentina desempenha uma função importante na integridade estrutural de células e tecidos (NIEMINEM *et al*., 2006), na adesão, na migração (GONZALES *et al*., 2001; HOMAN *et al*., 1998; TSURUTA e JONES,

2003; KREIS *et al*., 2005), na transdução de sinal (JANOSCH *et al*., 2000; IVASKA *et al*., 2005; PERLSON *et al*., 2005; KUMAR *et al*., 2007) e em processos apoptóticos (YANG *et al*., 2005).

Os FI sofrem alterações dinâmicas na sua organização citoplasmática durante diferentes estágios do ciclo celular ou em resposta a sinais celulares (STEINERT e ROOP, 1988; HELFAND *et al*., 2005) sendo a fosforilação um mecanismo central na regulação da organização da rede citoplasmática de FI (ERIKSSON e GOLDMAN, 1993).

I.7 Fosforilação

A fosforilação protéica é uma modificação pós-traducional importante para as vias de transdução de sinal que controla o metabolismo, crescimento, divisão e diferenciação celular, motilidade, tráfego de organelas, transporte de membrana, contração muscular, imunidade, memória e aprendizado (MANNING *et al*., 2002a,b).

Essa modificação covalente tem a propriedade de regular a função de proteínas em resposta a estímulos extracelulares (NESTLER e GREENGARD, 1999). Segundos mensageiros intracelulares, tais como o AMPc e o cálcio regulam funções neuronais através de alteração no estado de fosforilação de proteínas intracelulares. A fosforilação altera a função proteica de uma maneira rápida e reversível. O grau de fosforilação de uma proteína alvo reflete um balanço entre as ações contrárias de proteínas cinases e fosfatases, integrando um conjunto de rotas de sinalização celular (Figura 3). Entre o substrato de cinases e fosfatases estão enzimas, receptores de

neurotransmissores, canais iônicos e proteínas estruturais (PURVES *et al.*, 2005).

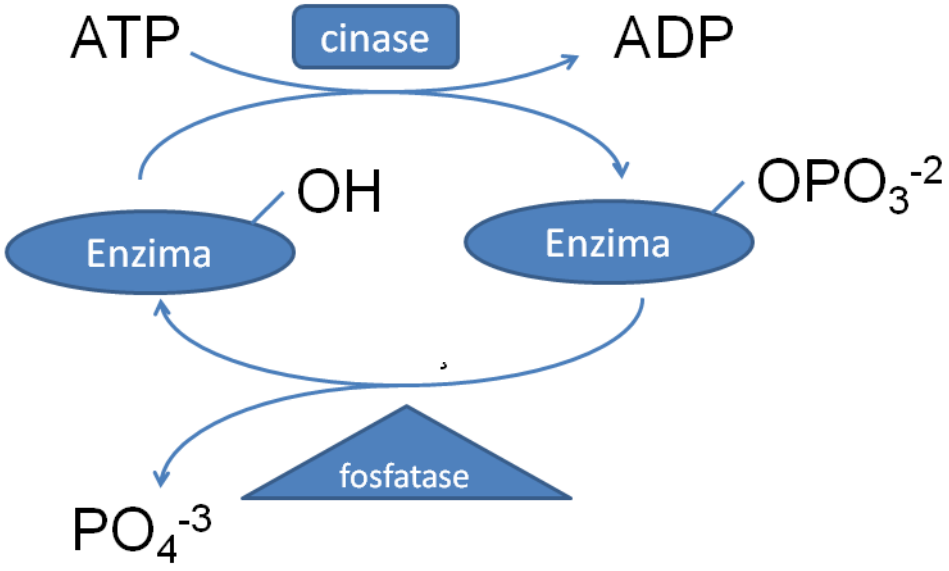


Figura 3 – Regulação das proteínas celulares por fosforilação. Proteínas cinases transferem grupos fosfato do ATP para resíduos de serina, treonina ou tirosina em um substrato protéico. A remoção dos grupos fosfato é catalisada por proteínas fosfatases (adaptado de Purves e colaboradores, 2005).

Os componentes do citoesqueleto estão entre as principais proteínas alvo modificadas por fosforilação em resposta a sinais extracelulares que podem determinar a morfologia neuronal (SANCHEZ *et al.*, 2000). Nos FI a fosforilação é o principal mecanismo responsável pela sua modulação, contribuindo na sua organização e função de maneira célula e tecido específica (GRANT e PANT, 2000; OMARY *et al.*, 2006).

Os FI podem ser fosforilados nas regiões aminoterminal e/ou carboxiterminal. A fosforilação do domínio aminoterminal está relacionada com a capacidade de associação e desassociação dos FI, sendo controlada por

cinases dependentes de segundo mensageiros (SIHAG *et al*., 2007). Já no domínio carboxiterminal a fosforilação é realizada por cinases independentes de segundo mensageiros e tem implicações na interação dos FI com outras estruturas do citoesqueleto (CHOU *et al*., 1997; GRANT e PANT, 2000).

Os FI são substratos de inúmeras serina/treonina cinases incluindo a proteína cinase C (PKC), a proteína cinase dependente de cálcio e calmodulina (PKCaMII), a proteína cinase dependente de AMPc (PKA), a família das proteínas cinases ativadas por mitógeno (MAPK), a proteína cinase dependente de ciclina 5 (CDK5), entre outras. Estas cinases possuem um papel significativo na regulação da estrutura e associação dos FI, bem como na interação deles com outras proteínas (ERIKSSON *et al*., 2004; SIHAG *et al*., 2007). Além disso, a ação dessas cinases nos FI é sítio específica, ou seja, elas fosforilam um resíduo específico dentro da estrutura protéica (SIHAG *et al*., 2007). Proteínas serina/treonina fosfatases como a proteína fosfatase 1 (PP1) e as proteínas fosfatases 2A (PP2A) e 2B (PP2B ou calcineurina) também desempenham um papel importante na regulação do citoesqueleto (GRANT e PANT, 2000).

O estado de fosforilação dos NF tem um papel importante no controle da integridade do citoesqueleto, no transporte e no diâmetro axonal (MOTIL *et al*., 2006; SIHAG *et al*., 2007; STRACK *et al*., 1997), além de promover a interação desses FI com as mitocôndrias (WAGNER *et al*., 2003). Também pode modular a função de organelas (TOIVOLA *et al*., 2005) e proteger os NF da proteólise (GRANT e PANT, 2000). Os NF são altamente fosforilados *in vivo*, porém o grau de fosforilação do NFM e do NFH e, especialmente do NFL, é

diferente em cada compartimento neuronal sendo altamente fosforilados no axônio, mas pouco fosforilados no corpo celular e nos dendritos (GOTOW e TANAKA, 1994; GOTOW *et al*., 1994).

A região amino-terminal dos NF é fosforilada em sítios distintos na NFL e NFM. Os sítios mais frequentes de fosforilação dessas subunidades são os resíduos Ser55 na NFL fosforilados pela PKA e pela PKC e PKA nos resíduos Ser23 da NFM (SIHAG e NIXON, 1990; SIHAG *et al*., 1999). Além disso, estudos *in vitro* mostraram que os resíduos Ser57 e Ser51 da NFL são substratos da PKCaMII e PKC, respectivamente (GONDA *et al*., 1990; HASHIMOTO *et al*., 2000).

A fosforilação da região carboxi-terminal das subunidades NFM e NFH dos FI ocorre em uma sequencia de repetições de lisina, serina e prolina (repetições do tipo KSP) (GEISLER *et al*., 1987; XU *et al*., 1992; LEE *et al*., 1988). A fosforilação desses sítios pode ser regulada por cascatas de transdução de sinal disparadas por fatores de crescimento, influxo de cálcio ou neurotoxinas, sendo realizadas por cinases como as MAPK e a CDK5 que são direcionadas por prolina para fosforilar a serina (LI *et al*., 1999 a,b; PIEROZAN *et al*., 2012).

Assim como nos NF, a polimerização de GFAP e da vimentina também é regulada pela fosforilação/desfosforilação do domínio aminoterminal que contém muitos sítios fosforiláveis (INAGAKI *et al*., 1996; GOHARA *et al*., 2001; TAKEMURA *et al*., 2002). Para a GFAP foram identificados seis diferentes sítios de fosforilação Thr7, Ser8, Ser13, Ser17 e Ser34 na região aminoterminal e Ser389 na região carboxiterminal (INAGAKI *et al*., 1990; NAKAMURA *et al*.,

1992; TSUJIMURA *et al*., 1994; SEKIMATA *et al*., 1996), entretanto pouco se sabe a respeito do papel de cada um desses sítios na associação, organização estrutural e disposição dos FI. A fosforilação/desfosforilação da região amino-terminal da GFAP em aminoácidos específicos regula a associação dessa proteína e é importante para a sua distribuição durante o ciclo celular (RALTON *et al*., 1994; YASUI *et al*., 1998; KAWAJIRI *et al*., 2003). Além disso, sugere-se que a fosforilação da GFAP regula a plasticidade estrutural dos filamentos gliais e eventualmente as funções dos astrócitos e protege a GFAP da degradação (TAKEMURA *et al*., 2002; KOROLAINEN *et al*., 2005). Já a vimentina apresenta vários sítios específicos de fosforilação na região amino-terminal. Essa modificação pós-traducional nessa proteína parece ter um papel importante na migração celular (IVASKA *et al*., 2005).

A fosforilação/desfosforilação é o principal mecanismo regulatório dos FI e de outras proteínas do citoesqueleto e alterações nos níveis fisiológicos de fosforilação/desfosforilação dessas proteínas são consideradas eventos críticos em patologias do SNC podendo levar a disfunção neurológica e morte celular (MILLER *et al*., 2002). Várias doenças neurodegenerativas, tais como a doença de Alzheimer, a doença de Parkinson e a doença de Huntington são caracterizadas por acúmulo de agregados de filamentos insolúveis no citoplasma das células (GOEDERT, 1998; JULIEN, 1999; DiPROSPERO *et al*., 2004), os quais estão relacionados à desregulação na atividade de cinases e fosfatases aos FI, constituindo-se em um mecanismo na neurodegeneração (PETZOLD, 2005).

Assim, no presente trabalho investigamos os efeitos dos principais ácidos orgânicos acumulados na HMGA sobre a homeostase redox em estriado de ratos jovens, sobre a resposta inflamatória e a homeostase redox em astrócitos de córtex cerebral de ratos neonatos e sobre a fosforilação de proteínas do citoesqueleto em estriado e córtex cerebral ratos jovens. Dividimos esse trabalho em três capítulos que correspondem a três artigos científicos, o primeiro já aceito e publicado e outros dois submetidos.

II OBJETIVOS

II.1 Objetivo geral

O objetivo geral desse trabalho foi o de explorar alguns dos mecanismos patogênicos dos principais ácidos orgânicos acumulados na acidúria 3-hidroxi-3-metilglutárica que possam explicar ao menos em parte o dano cortical e estriatal característico dos pacientes afetados por essa doença, focando na homeostase redox, resposta inflamatória e fosforilação de proteínas do citoesqueleto de ratos jovens.

II.2 Objetivos específicos

- Investigar os efeitos *ex vivo* da administração intraestriatal dos ácidos HMG e MGA sobre o dano oxidativo lipídico (níveis de substâncias reativas ao ácido tiobarbitúrico), dano oxidativo proteico (formação de carbonilas), defesas antioxidantes não-enzimáticas (concentrações de glutationa reduzida), formação de espécies reativas (oxidação da 2',7'-diclorofluorescina e determinação de nitritos e nitratos) e enzimáticas (atividades das enzimas antioxidantes glutationa peroxidase, glutationa redutase, superóxido dismutase, catalase e glicose-6-fosfato desidrogenase), em estriado de ratos de 30 dias de vida.
- Investigar os efeitos *in vitro* dos ácidos HMG e MGA sobre a formação de espécies reativas (oxidação da 2',7'-diclorofluorescina), as defesas antioxidantes não-enzimáticas (concentrações de glutationa reduzida), a viabilidade celular (formação do formazan e incorporação do iodeto de propídeo), bem como sobre a resposta inflamatória (concentrações de IL-1β,

IL-6, TNFα e NFκB) e a indução de gliose reativa (quantificação de GFAP) em astrócitos de córtex cerebral de ratos neonatos cultivados (1 a 2 dias de vida).

- Investigar os efeitos *in vitro* dos ácidos HMG, MGA e MGT sobre o sistema de fosforilação dos FI (incorporação de [^{32}P]-ortofosfato de sódio, proteínas cinases e fosfatases) de astrócitos (GFAP) e neurônios (NFL, MFM e NFH), bem como, investigar os mecanismos de ação dos efeitos desencadeados pelos metabólitos testados em estriado e córtex cerebral de ratos de 30 dias de vida.

PARTE II

Artigos Científicos

Capítulo 1

In vivo experimental evidence that the major metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency induce oxidative stress in striatum of developing rats: A potential pathophysiological mechanism of striatal damage in this disorder

Carolina Gonçalves Fernandes, Mateus Struecker da Rosa, Bianca Seminotti, Paula Pierozan, Rafael Wolter Martell, Valeska Lizzi Lagranha, Estela Natacha Brandt Busanello, Guilhian Leipnitz, Moacir Wajner

Artigo Científico publicado no periódico

Molecular Genetics and Metabolism

Molecular Genetics and Metabolism 109 (2013) 144–153

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

In vivo experimental evidence that the major metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency induce oxidative stress in striatum of developing rats: A potential pathophysiological mechanism of striatal damage in this disorder

Carolina Gonçalves Fernandes [a], Mateus Struecker da Rosa [a], Bianca Seminotti [a], Paula Pierozan [a], Rafael Wolter Martell [a], Valeska Lizzi Lagranha [a], Estela Natacha Brandt Busanello [a], Guilhian Leipnitz [a], Moacir Wajner [a,b,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 26 February 2013
Received in revised form 29 March 2013
Accepted 29 March 2013
Available online 6 April 2013

Keywords:
3-Hydroxy-3-methylglutaric aciduria
3-Hydroxy-3-methylglutaric acid
3-Methylglutaric acid
Oxidative stress
Striatum

ABSTRACT

3-Hydroxy-3-methylglutaryl-CoA lyase (HL) deficiency is a genetic disorder biochemically characterized by predominant accumulation of 3-hydroxy-3-methylglutaric (HMG) and 3-methylglutaric (MGA) acids in tissues and biological fluids of affected individuals. Clinically, the patients present neurological symptoms and basal ganglia injury, whose pathomechanisms are partially understood. In the present study, we investigated the *ex vivo* effects of intrastriatal administration of HMG and MGA on important parameters of oxidative stress in striatum of developing rats. Our results demonstrate that HMG and MGA induce lipid and protein oxidative damage. HMG and MGA also increased 2′,7′-dichlorofluorescein oxidation, whereas only HMG elicited nitric oxide production, indicating a role for reactive oxygen (HMG and MGA) and nitrogen (HMG) species in these effects. Regarding the enzymatic antioxidant defenses, both organic acids decreased reduced glutathione concentrations and the activities of superoxide dismutase and glutathione reductase and increased glutathione peroxidase activity. HMG also provoked an increase of catalase activity and a diminution of glucose-6-phosphate dehydrogenase activity. We finally observed that antioxidants fully prevented or attenuated HMG-induced alterations of the oxidative stress parameters, further indicating the participation of reactive species in these effects. We also observed that MK-801, a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor, prevented some of these effects, indicating the involvement of the NMDA receptor in HMG effects. The present data provide solid evidence that oxidative stress is induced *in vivo* by HMG and MGA in rat striatum and it is presumed that this pathomechanism may explain, at least in part, the cerebral alterations observed in HL deficiency.

1. Introduction

3-Hydroxy-3-methylglutaryl-CoA lyase (HL) deficiency (OMIM 246450) is an autosomal recessive genetic disorder biochemically characterized by predominant tissue accumulation and high urinary excretion of large quantities of 3-hydroxy-3-methylglutarate (HMG) and 3-methylglutarate (MGA), as well as 3-methylglutaconate, 3-hydroxyisovalerate and 3-methylcrotonylglycine to lesser amounts [1–3].

Clinical presentation usually occurs in the first year of life. Acute episodes are common and are characterized by vomiting, diarrhea, dehydration, hypotonia, hypothermia, lethargy, coma and apnea, as well as by metabolic acidosis and hypoketotic hypoglycemia. Other signs include macrocephalia, developmental delay, hepatomegalia with liver test alterations, acute pancreatitis and dilated cardiomyopathy [2,4–7]. This disease is fatal in approximately 20% of cases, although

Abbreviations: CAT, catalase; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichloroflurescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DNPH, 2,4-dinitrophenylhydrazine; GC/MS, gas chromatography/mass spectrometry; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HL, 3-hydroxy-3-methylglutaryl-CoA lyase; HMG, 3-hydroxy-3-methylglutaric acid; HMGA, 3-hydroxy-3-methylglutaric aciduria; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MEL, melatonin; MGA, 3-methylglutaric acid; NAC, N-acetylcysteine; NMDA, N-methyl-D-aspartate; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SPSS, statistical package for the social sciences; TBA-RS, thiobarbituric acid- reactive substances; TCA, trichloroacetic acid.

* Corresponding author at: Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos N° 2600 – Anexo, CEP 90035-003, Porto Alegre, RS – Brazil. Fax: +55 51 3308 5535.
E-mail address: mwajner@ufrgs.br (M. Wajner).

1096-7192/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymgme.2013.03.017

the symptoms tend to be milder after childhood [8,9]. Cerebral magnetic resonance neuroimaging shows predominantly abnormalities in the caudate nucleus and dentate nucleus and multiple coalescent lesions in periventricular subcortical white matter and arcuate fibers [10–12].

The mechanisms underlying the pathogenesis of the brain damage in HL deficiency are poorly known. However, it has been suggested that hypoglycemia associated with deficit of ketone bodies, secondary carnitine deficiency and intracellular accumulation of toxic organic acids may be involved in the pathophysiology of this disease [13–15]. In this context, previous *in vitro* studies demonstrated that HMG and MGA induce lipid and protein oxidative damage and reduce the non-enzymatic antioxidant defenses in cerebral cortex, striatum and liver of young rats [16–18]. It was also shown that MGA induces mitochondrial dysfunction and inhibits Na^+,K^+-ATPase activity in synaptosomes prepared from rat brain [19]. However, to the best of our knowledge, there is no study on the *in vivo* effects of the major metabolites accumulating in HL deficiency on redox cellular homeostasis.

Therefore, in the present work we investigated whether high striatum concentrations of HMG and MGA achieved by an acute intrastriatal injection of these organic acids to young rats could affect redox homeostasis in the hope to better clarify the pathomechanisms of striatal abnormalities observed in HL deficiency. For this purpose, important parameters of oxidative stress were determined, including malondialdehyde (MDA) concentrations (lipid peroxidation), carbonyl formation (protein oxidative damage), 2′,7′-dichlorofluorescin (DCFH) oxidation, nitric oxide production, reduced glutathione (GSH) concentrations (non-enzymatic antioxidant defenses) and the activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPx) and catalase (CAT).

2. Materials and methods

2.1. Reagents

All reagents used were of analytical grade and purchased from Sigma Co. (St. Louis, MO, USA). The solutions of HMG (4 M), MGA (4 M) and NaCl were prepared in water and the pH was adjusted to 7.4 with NaOH. N-acetylcysteine (NAC), melatonin (MEL), N^{ω}-nitro-L-arginine methyl ester (L-NAME), dizocilpine maleate (MK-801) and the combination of α-tocopherol (vitamin E) plus ascorbic acid (vitamin C) were dissolved in water, and the pH was similarly adjusted to 7.4.

2.2. Animals

Thirty-day-old male Wistar rats, obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, were used. The animals were maintained on a 12:12 h light/dark cycle (lights on 07.00–19.00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil and followed the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

2.3. Administration of 3-hydroxy-3-methylglutaric (HMG) and 3-methylglutaric acids (MGA)

Rats were anesthetized with an intraperitoneal (i.p.) injection of equitesine (3.33 mL/Kg), which consists of a mixture of 0.25 M chloral hydrate, 88 mM magnesium sulfate heptahydrate, 10 mg/mL sodium thiopental, 5.8 M propylene glycol and 1.97 M ethanol. They were thereafter placed on a stereotaxic apparatus. Two small holes were drilled in the skull and 1 μL (4 μmol) of a HMG, MGA or NaCl solution (control group) was slowly injected into each striatum over 3 min via a needle connected by a polyethylene tube to a 10 μL Hamilton syringe. The needle was left in place for another 1 min before being softly removed, so that the total procedure lasted 4 min. The coordinates for injections were as follows: 0.6 mm posterior to the bregma, 2.6 mm lateral to the midline and 4.5 mm ventral from dura [20]. In some experiments, animals were pre-treated i.p. with MEL (100 mg/kg), NAC (150 mg/kg) or vitamin E (40 mg/kg) plus vitamin C (100 mg/kg) for 3 days, after which they received an acute injection of HMG. In other experiments, MK-801 (6 nmol) or L-NAME (0.2 nmol) was injected intrastriatally 30 min before the administration of HMG.

Some animals aged 7 and 30 days of life were injected with a single dose of HMG or MGA (10 μmol/g body weight of each organic acid) and sacrificed 30 min after the administration. The concentrations of the organic acids were measured in striatum from these animals 30 min after injection.

2.4. Striatum preparation

The rats were sacrificed by decapitation without anesthesia 30 min after the intrastriatal injection of the organic acid or NaCl. We sacrificed the animals and collected the samples 30 min after the injection because we aimed to investigate short-lived effects (acute evaluation) of the major metabolites accumulating in 3-hydroxy-3-methylglutaric aciduria. The brain was rapidly excised on a Petri dish placed on an ice plate. The olfactory bulb, pons, medulla, cerebral cortex and cerebellum were discarded and the striatum was dissected, weighed, and homogenized in 4 (1:4, w/v) (measurement of nitric oxide production) or 10 volumes (1:10, w/v) (evaluation of the other oxidative stress parameters) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4 °C to discard nuclei and cell debris [21]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to measure oxidative stress parameters. Slices (400 μm) of striatum were also prepared and used for DCFH oxidation.

2.5. Organic acid analysis

HMG and MGA quantification in brain homogenates was performed by gas chromatography/mass spectrometry (GC/MS) according to Sweetman [22] with some modifications [23]. For this analysis, we used striatum homogenates (1:10 w/v in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl) prepared from rats that received a single intrastriatal administration of HMG (4 μmol) or MGA (4 μmol), as described previously. Brain homogenates previously incubated for 1 h at 37 °C with HMG (20 mM) or MGA (20 mM) to evaluate the metabolism of these organic acids in the brain. We also utilized striatum from 7- and 30-day-old rats injected i.p. with 10 μmol/g body weight of HMG or MGA, which was homogenized (1:10, w/v) in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. An aliquot of 500 μL of each sample (brain homogenates) was first acidified to pH 1.5–2.0 and mixed with 100 μL hexadecane (internal standard). Standard solutions of HMG and MGA at the concentration of 1 mM were also submitted to the same procedure.

2.6. Oxidative stress parameters

2.6.1. Malondialdehyde (MDA) concentrations

MDA concentrations were estimated by measuring thiobarbituric acid-reactive substances (TBA-RS) [24], with slight modifications. Briefly, 200 μL of 10% trichloroacetic acid (TCA) and 300 μL of 0.67%

thiobarbituric acid in 7.1% sodium sulfate were added to tissue supernatants containing 0.3 mg of protein and incubated for 2 h in a boiling water bath. The resulting pink-stained complex was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 nm and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane. MDA concentrations were calculated as nmol MDA/mg of protein.

2.6.2. Protein carbonyl formation

Protein carbonyl formation was measured spectrophotometrically according to Reznick and Packer [25]. Two hundred microliters of striatum supernatants containing 0.3 mg of protein were treated with 400 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 h. Samples were then precipitated with 600 μL of 20% TCA and centrifuged for 5 min at 10,000 *g*. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and suspended in 550 μL of 6 M guanidine prepared in 2.5 N HCl at 37 °C for 5 min. The absorbance was read at 365 nm. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 365 nm. The results were calculated as nmol of carbonyl groups/mg of protein, using the extinction coefficient of 22,000 × 10^6 nmol/mL for aliphatic hydrazones.

2.6.3. 2′,7′-Dichlorofluorescin (DCFH) oxidation

Reactive oxygen species (ROS) production was assessed by determining DCFH oxidation according to LeBel [26]. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl and incubated with tissue slices (30 mg) during 30 min at 37 °C. DCFH-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity parallels the amount of ROS formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (0.1–1 μM) and the levels of ROS were calculated as pmol DCF formed/mg protein.

2.6.4. Nitrate and nitrite content

Nitrate and nitrite concentrations were evaluated according to Navarro-Gonzálvez [27] with some modifications. One hundred and fifty microliters of tissue supernatants (containing approximately 1.2 mg of protein) were deproteinized by adding 125 μL of 75 mM $ZnSO_4$ solution, followed by centrifugation at 9000 *g* for 2 min at 25 °C. The supernatant obtained was neutralized with 55 mM NaOH solution and diluted in 5 volumes of glycine buffer solution, pH 9.7. Copper-coated cadmium granules (600–1000 mg) were added to the supernatants to convert all nitrate into nitrite in the biological samples. Aliquots of 200 μL were then treated with 200 μL of Griess reagent (2% sulfanilamide in 5% HCl and 0.1% N-1-(naphtyl)ethylenediamine in H_2O) and incubated at room temperature by 10 min. The absorbance was read at 505 nm. A calibration curve was prepared with $NaNO_2$ at concentrations ranging from 1 to 125 μM. The final results were expressed in μmol of nitrate and nitrite/mg of protein.

2.6.5. Reduced glutathione (GSH) concentrations

GSH concentrations were measured according to Browne and Armstrong [28] with slight modifications. One hundred and eighty-five microliters of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and 15 μL of o-phthaldialdehyde (1 mg/mL) were added to 30 μL of sample (0.3–0.5 mg of protein) previously deproteinized with metaphosphoric acid. This mixture was incubated at room temperature in a dark room for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.001–1 mM) and the concentrations were calculated as nmol/mg of protein.

2.6.7. Superoxide dismutase (SOD) activity

SOD activity was assayed according to Marklund [29]. The reaction medium contained 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase, 0.38 mM pyrogallol and approximately 1 μg of protein. The inhibition of autoxidation of pyrogallol, a process dependent on superoxide, occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The results are reported as U/mg of protein.

2.6.8. Glutathione reductase (GR) activity

GR activity was measured according to Carlberg and Mannervik [30]. The enzyme activity was determined by monitoring the NADPH decrease at 340 nm in a medium with 200 mM sodium phosphate buffer, pH 7.5, containing 6.3 mM ethylenediaminetetraacetic acid, 1 mM GSSG, 0.1 mM NADPH and tissue supernatants (approximately 3 μg of protein). The specific activity was calculated and expressed as U/mg of protein.

2.6.9. Glucose-6-phosphate dehydrogenase (G6PD) activity

G6PD activity was measured by the method of Leong and Clark [31], in which the reaction mixture contained 100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$ and approximately 3 μg of protein. The reaction was started by the addition of 1 mM glucose-6-phosphate and was followed in a spectrophotometer at 340 nm. The specific activity is represented as U/mg of protein.

2.6.10. Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel [32] using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH decrease at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM EDTA, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH and approximately 3 μg of protein. The specific activity was calculated as U/mg of protein.

2.6.11. Catalase (CAT) activity

CAT activity was assayed according to Aebi [33] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and approximately 1 μg of protein. The specific activity was calculated as U/mg of protein.

2.7. Protein content

The protein content was determined by the method of Lowry et al. [34], using bovine albumin as standard.

2.8. Statistical analysis

Data were expressed as means ± SD for absolute values. Assays were performed in duplicate or triplicate and the mean was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F value was significant. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. HMG and MGA concentrations achieved in rat striatum

We first determined the concentrations of HMG and MGA achieved in the striatum after a single injection of each of the organic acids. We verified that mean HMG and MGA concentrations achieved in the striatum were respectively 140 and 200 μM.

Next we tested *in vitro* whether 1) HMG could be converted to MGA by measuring MGA formation from HMG in brain homogenates and 2) MGA could be metabolized in the brain. We observed under our conditions that MGA could not be detected when HMG was incubated for 1 h with brain homogenates and that MGA was not metabolized in the brain since the amount of MGA remained the same when brain was exposed for 1 h with this organic acid (results not shown). These data suggest that the brain does not contain the enzymes necessary for HMG and MGA metabolism as it was previously reported [35] and indicate that our results were due to true effects of HMG and MGA injected into the striatum.

We also investigated whether HMG and MGA are able to cross the blood–brain barrier (BBB) by i.p. injection of each organic acid (10 μmol/g body weight) to 7- and 30-day-old (young) rats. We barely detected both organic acids in the striatum of 30-day-old rats (concentrations around 20 μM). However, the same dose of these metabolites injected i.p. in 7-day-old rats gave rise to high striatum accumulation of these compounds (HMG: 348 μM; MGA; 480 μM), implying that the immature BBB is much more permeable to these organic acids.

3.2. HMG and MGA intrastriatal administration induces lipid and protein oxidative damage in rat striatum

Fig. 1A shows that HMG and MGA [$F_{(2,13)} = 12.78$; $P < 0.001$] significantly increased MDA levels in rat striatum, indicating that these organic acids induce lipid oxidative damage.

We also evaluated the effect of intrastriatal administration of HMG and MGA on carbonyl formation, a product of protein oxidation, in rat striatum. We observed that HMG and MGA [$F_{(2,10)} = 8.13$; $P < 0.01$] significantly increased carbonyl formation (Fig. 1B), indicating that both organic acids provoke protein oxidative damage.

3.3. HMG and MGA intrastriatal administration increases reactive species production in rat striatum

We assessed whether reactive species were involved in HMG and MGA-induced pro-oxidant effects by investigating the influence of these organic acids on DCFH oxidation and nitrate plus nitrite production. We verified that HMG and MGA significantly increased DCFH oxidation [$F_{(2,13)} = 16.4$; $P < 0.001$] (Fig. 1C), whereas only HMG increased nitrate and nitrite production [$F_{(2,11)} = 2.97$; $P < 0.05$] (Fig. 1D).

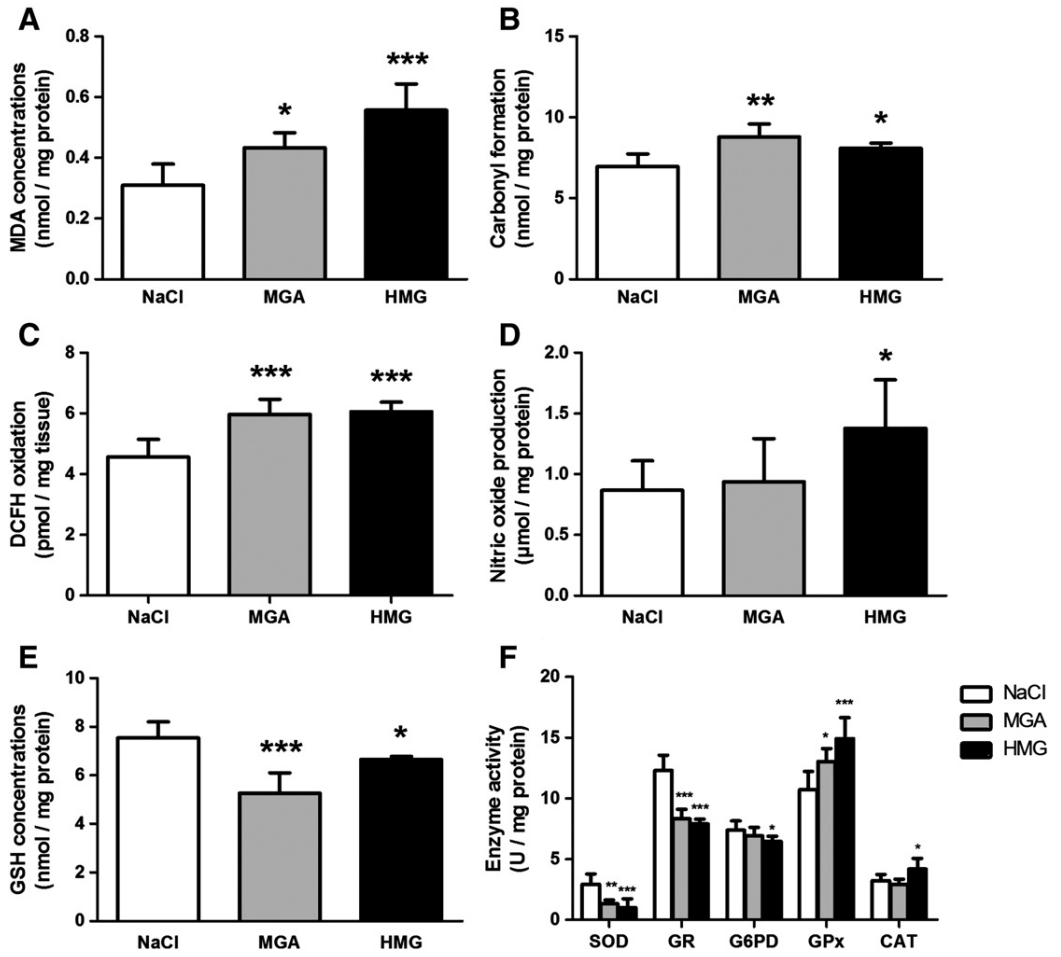


Fig. 1. Effect of intrastriatal administration of 3-hydroxy-3-methylglutaric acid (HMG, 4 μmol) or 3-methylglutaric acid (MGA, 4 μmol) on malondialdehyde (MDA) levels (A), carbonyl formation (B), 2′,7′-dichlorofluorescin (DCFH) oxidation (C), nitric oxide production (D), GSH concentrations (E), and the activities of superoxide dismutase (SOD), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPx) and catalase (CAT) (F) in rat striatum 30 min after injection. Data are expressed as mean ± SD of 5 to 6 independent experiments (animals) performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to rats that received intrastriatal NaCl injection (Duncan multiple range test).

3.4. HMG and MGA intrastriatal administration reduces GSH concentrations in rat striatum

The next step of this work was to investigate the effects of HMG and MGA on GSH concentrations. Fig. 1E depicts that HMG and MGA [$F_{(2,15)} = 20.29$; $P < 0.001$] decreased GSH concentrations in rat striatum, indicating a decrease of striatal non-enzymatic antioxidant defenses.

3.5. HMG and MGA intrastriatal administration modulates antioxidant enzyme activities in rat striatum

We then determined the effects of intrastriatal administration of HMG and MGA on the activities of the antioxidant enzymes SOD, GR, G6PD, GPx and CAT. It can be observed in Fig. 1F that HMG and MGA significantly decreased SOD [$F_{(2,15)} = 13.78$; $P < 0.001$] and GR [$F_{(2,15)} = 44.97$; $P < 0.001$] activities, whereas HMG decreased the activity of G6PD [$F_{(2,15)} = 2.88$; $P < 0.05$] in rat striatum. On the other hand, GPx activity was significantly increased by HMG and MGA intrastriatal administration [$F_{(2,15)} = 12.58$; $P < 0.01$], whereas HMG increased CAT activity [$F_{(2,15)} = 6.44$; $P < 0.05$] (Fig. 1F).

3.6. Antioxidants and MK-801 prevented HMG-induced alterations of the parameters of oxidative stress

We also investigated the effects of i.p. pre-treatment of MEL (Fig. 2), NAC (Fig. 3) and the combination of vitamin E plus vitamin C (Fig. 4) on HMG-induced lipid oxidative damage and alterations of the antioxidant defenses in rat striatum. The figures demonstrate that these antioxidants fully prevented or attenuated the effects of HMG on MDA levels (Figs. 2A, 3A and 4A), GSH concentrations (Figs. 2B, 3B and 4B) and the activities of SOD, GR, G6PD, GPx and CAT (Figs. 2C, 3C and 4C).

We then evaluated the influence of a single intrastriatal injection of the non-competitive NMDA receptor antagonist MK-801 and of the nitric oxide synthase (NOS) inhibitor L-NAME on the effects elicited by HMG (Fig. 5). We observed that MK-801 fully prevented lipid peroxidation induced by HMG (Fig. 5A), whereas L-NAME totally prevented and MK-801 attenuated GSH decrease caused by HMG (Fig. 5B).

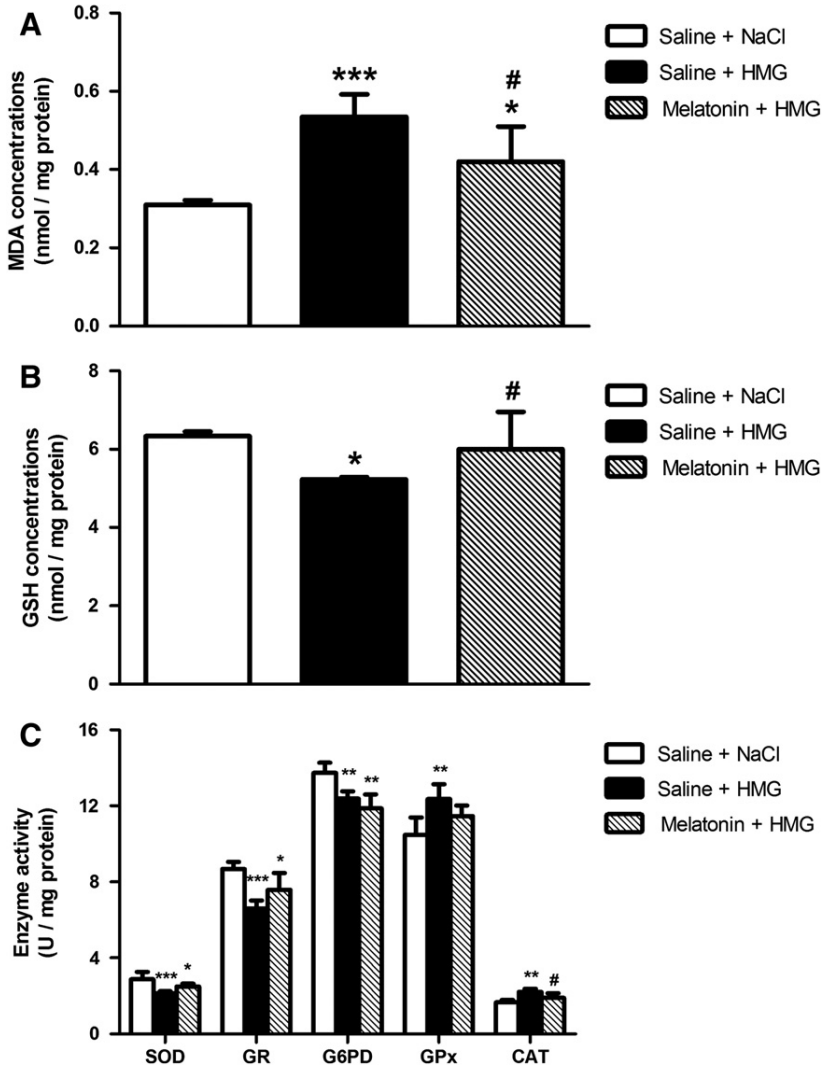


Fig. 2. Effect of melatonin (MEL; 100 mg/kg) on 3-hydroxy-3-methylglutaric acid (HMG, 4 μmol)-induced alterations on malondialdehyde (MDA) levels (A), glutathione (GSH) concentrations (B) and activities of antioxidant enzymes (C). Values are means ± standard deviation of five to six independent experiments (animals) performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control (NaCl-injected rats); $^{\#}P < 0.05$, compared to HMG (Duncan multiple range test).

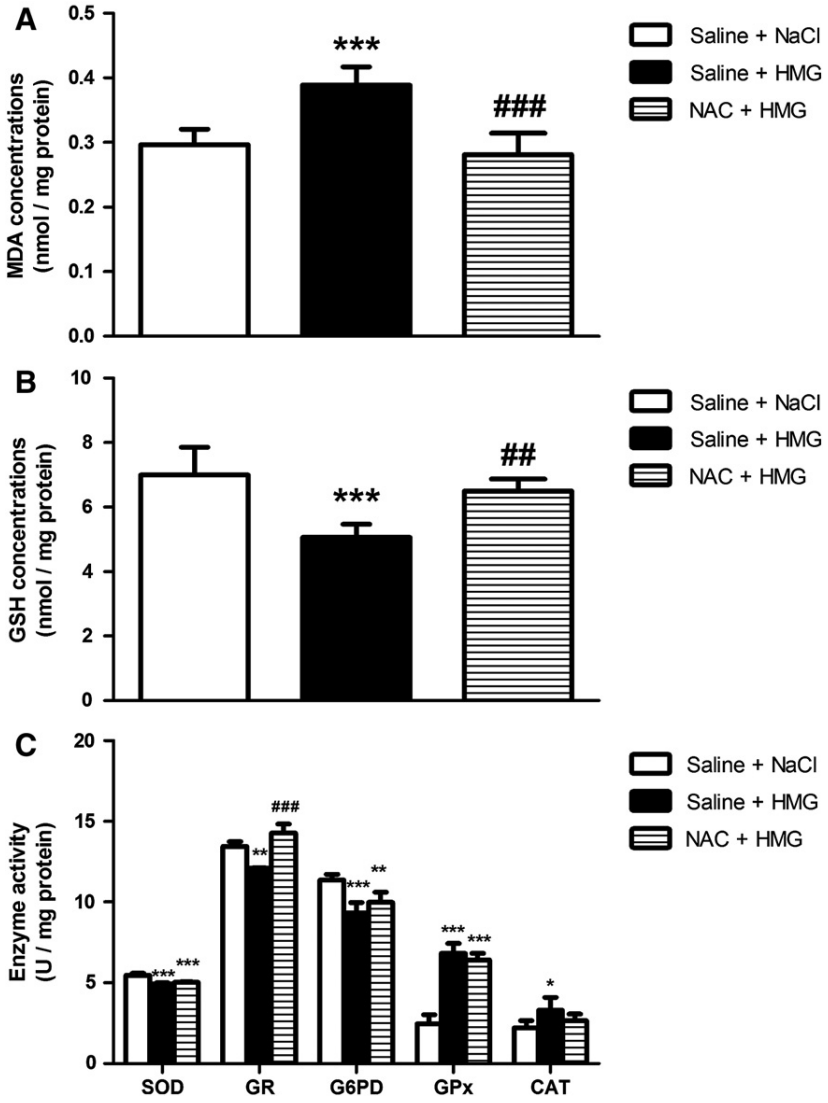


Fig. 3. Effect of N-acetylcysteine (NAC; 150 mg/kg) on 3-hydroxy-3-methylglutaric acid (HMG, 4 μmol)-induced alterations on malondialdehyde (MDA) levels (A), glutathione (GSH) concentrations (B) and activities of antioxidant enzymes (C). Values are means ± standard deviation of five to six independent experiments (animals) performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control (NaCl-injected rats); $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to HMG (Duncan multiple range test).

Regarding the enzymatic antioxidant defenses, L-NAME and MK-801 prevented the HMG-elicited decrease of SOD activity (Fig. 5C), whereas MK-801 avoided the decrease of the activity of GR and L-NAME barred the decrease of G6PD provoked by HMG (Fig. 5C).

4. Discussion

The mechanisms of brain injury in HL deficiency are poorly known, although it is conceivable that the hypoglycemia and/or hyperammonemia occurring during crises may be involved in the neurological dysfunction presented by the patients [2,7,35]. It could be also presumed that chronic accumulation of HMG and MGA may represent an important pathomechanism contributing to the cerebral symptoms and abnormalities observed in these patients, particularly during episodes of metabolic decompensation, in which the concentrations of these metabolites dramatically increase. Therefore, it seems relevant to investigate the *in vivo* role of the major metabolites accumulating in HL deficiency on the cell redox homeostasis in the brain in order to clarify the relationship between the clinical features and the biochemical abnormalities in this disorder.

In the present study we evaluated the effects of an acute intrastriatal administration of HMG and MGA on important parameters of oxidative stress in rat striatum, a cerebral structure mainly altered in the brain of the affected patients [10–12]. We found that intrastriatal administration of HMG and MGA induced lipid peroxidation in striatum, corroborating our previous *in vitro* findings showing that both organic acids increased TBA-RS levels in rat brain [18]. Since TBA-RS measurement reflects the amount of MDA generated, a final product formed by the oxidation of unsaturated fatty acids of lipid constituents of the tissues, these results indicate that HMG and MGA induced lipid oxidative damage *in vivo*. We also showed that the antioxidants MEL, NAC, the combination of vitamins C and E prevented or attenuated MDA concentrations increase elicited by HMG, implying that this effect was secondary to elevated reactive species generation, mainly hydroxyl and peroxyl radicals, which are scavenged by these antioxidants [36–38].

HMG and MGA also increased carbonyl formation in the striatum. In this context, it should be noted that carbonyl groups are generated in proteins by the oxidation of amino acid side chain residues (Pro, Arg, Lys, and Thr) mediated by reactive species [39]. We cannot also exclude the possibility that aldehydes resulting from lipid peroxidation, which were increased by HMG, may have secondarily induced carbonyl

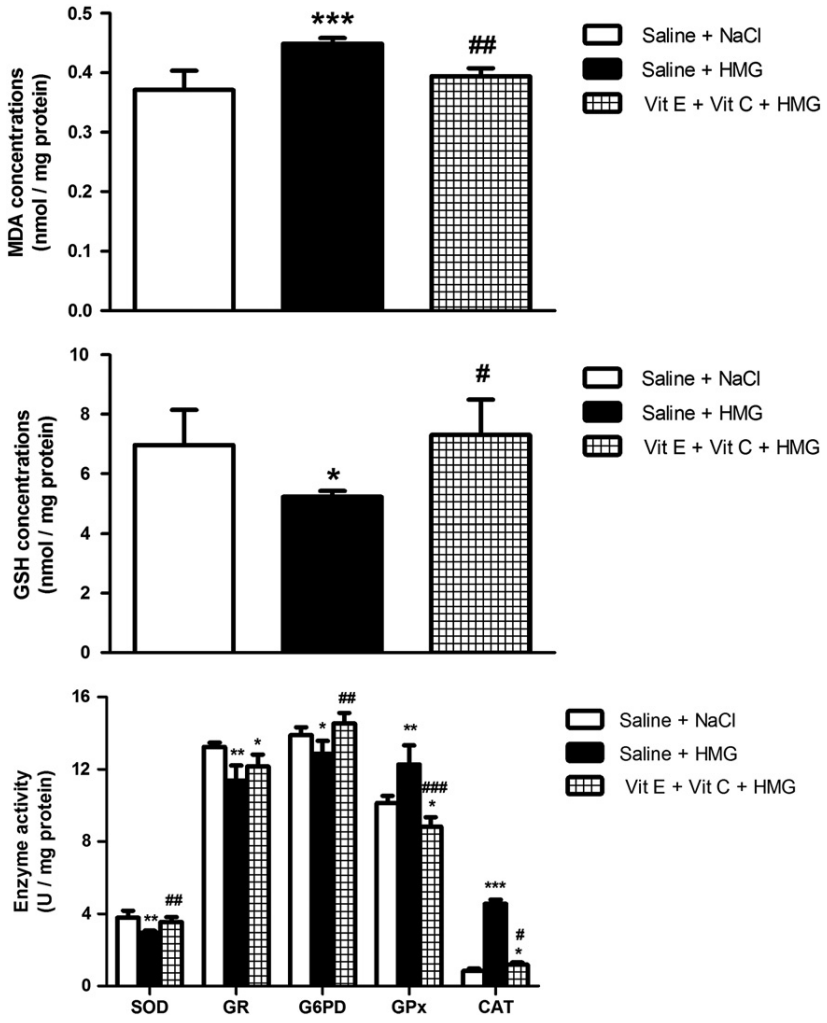


Fig. 4. Effect of the combination of α-tocopherol (vitamin E, Vit E; 40 mg/kg) and ascorbic acid (vitamin C, Vit C; 100 mg/kg) on 3-hydroxy-3-methylglutaric acid (HMG, 4 μmol)-induced alterations on malondialdehyde (MDA) levels (A), glutathione (GSH) concentrations (B) and activities of antioxidant enzymes (C). Values are means ± standard deviation of five to six independent experiments (animals) performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control (NaCl-injected rats); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to HMG (Duncan multiple range test).

generation [39]. Since carbonyl group generation is currently used as a marker of free radical-mediated protein oxidation [40], it is presumed that these organic acids provoked protein oxidative damage in the striatum.

We also verified that HMG and MGA increased DCFH oxidation, suggesting the involvement of ROS in HMG and MGA pro-oxidant effects [41,42]. HMG also increased nitric oxide production, as determined by the augmented formation of nitrates and nitrites provoked by this metabolite. These data, allied to our findings showing that NAC, MEL, the combination of vitamins C and E and L-NAME (NOS inhibitor) prevented lipid peroxidation and also the alterations of antioxidant defenses elicited by HMG, reinforce the presumption that the oxidative damage was due to increased generation of reactive oxygen and nitrogen species. On the other hand, it is possible that the increase of reactive nitrogen species (RNS) induced by HMG was partly due to overstimulation of NMDA receptors since MK-801, the antagonist of these receptors, also prevented HMG-induced pro-oxidant effects [43].

Regarding the antioxidant system, we found that HMG and MGA significantly reduced GSH concentrations, and this is in line with our previous *in vitro* findings [18]. Considering that endogenous GSH is the main naturally-occurring antioxidant in the brain and is used to evaluate the capacity of a tissue to prevent the damage associated to free radical processes [38], it is presumed that the rat striatum non-enzymatic antioxidant defenses were compromised by HMG and MGA *in vivo*. Moreover, since GSH is considered an important defense against lipid oxidative damage scavenging reactive species responsible for the initiation of this process, it is feasible that lipid peroxidation elicited by HMG and MGA could be secondary to GSH reduction. It is emphasized that GSH reduction could be due to the increase of reactive species elicited by HMG and MGA, which may have consumed this important brain antioxidant, reducing its final concentrations.

HMG and MGA also significantly decreased SOD and GR activities and increased GPx activity in rat striatum. In addition, HMG caused a significant increase of CAT activity and a decrease of G6PD activity. Although we cannot at present explain the mechanisms by which SOD activity was reduced, it has been suggested that a decrease in the activities of antioxidant enzymes, such as SOD, may be due to ROS causing a site-specific amino acid modification [43,44]. We should emphasize that a reduction of SOD activity may lead to superoxide anions excess that usually generates other forms of carbon-, nitrogen- and oxygen-centered radicals that could contribute to the lipid and protein oxidative damage induced by HMG and MGA in the brain. Furthermore, the increase of GPx activity, DCFH oxidation and lipid peroxidation caused by HMG and MGA administration suggest that hydrogen peroxide and lipid peroxide are probably involved in HMG- and MGA-induced

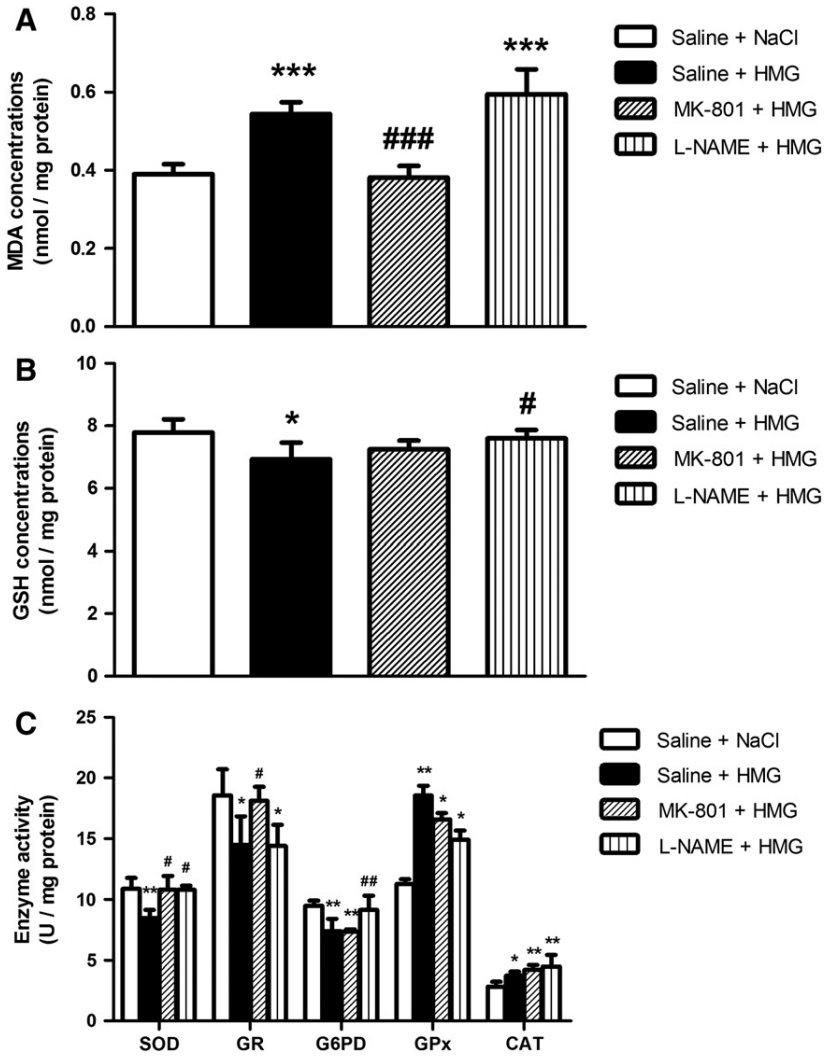


Fig. 5. Effect of dizocilpine maleate (MK-801; 6 nmol) or N^{ω}-nitro-L-arginine methyl ester (L-NAME; 0.2 nmol) on 3-hydroxy-3-methylglutaric acid (HMG, 4 μmol)-induced alterations on malondialdehyde (MDA) levels (A), glutathione (GSH) concentrations (B) and activities of antioxidant enzymes (C). Values are means ± standard deviation of five to six independent experiments (animals) performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control (NaCl-injected rats); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to HMG (Duncan multiple range test).

oxidative damage in rat striatum. As regards the increased CAT activity, induction of the expression of this antioxidant enzyme at the gene level might have taken place as a compensatory mechanism in response to increased formation of hydrogen peroxide [45–47] induced by HMG. Finally, it is feasible that HMG-induced diminution of G6PD activity may have contributed to the decreased GR activity due to the lack of NADPH for GSH recycling through this enzyme.

We also found that the non-competitive NMDA receptor antagonist MK-801 prevented the lipid oxidation and the alterations of antioxidant defenses caused by HMG. These data highlight a role for NMDA receptor in these effects. The fact that NMDA receptors may be involved in HMG-induced effects is not surprising because of the structural similarity between HMG and glutamate. Therefore, it may be of interest to study whether these organic acids and especially HMG interact with NMDA receptors. On the other hand, we cannot exclude the possibility that induction of NOS activity leading to generation of RNS was at least partially due to overstimulation of NMDA glutamate receptors by this organic acid [38,43,48].

Since oxidative stress results from an imbalance between the total antioxidant defenses and the reactive species generated in a tissue, our present data strongly indicate that HMG and MGA induce oxidative stress *in vivo* in rat striatum. It must be emphasized here that ROS are capable to oxidize different molecules, including lipids, proteins, sugars and DNA, representing a key event in the pathogenic cascade leading to necrotic or apoptotic cell death [49–51]. Furthermore, oxidative stress is a very deleterious condition especially to the brain compared with other tissues [38]. This is supported by the fact that the brain has a high rate of oxidative metabolism coupled to ROS production, lower activity of antioxidant enzymes, reduced content of non-enzymatic antioxidants and higher peroxidation potential because of its high content of polyunsaturated fatty acids [38].

Currently, we cannot determine the pathophysiological relevance of the present data since to our knowledge brain concentrations of HMG and MGA were not yet established in HL deficiency. However, it should be stressed that the significant alterations of the biochemical parameters were obtained at micromolar concentrations in our present *in vivo* model. On the other hand, we found that HMG and MGA were shown to rapidly cross the BBB in 7-day-old rats in the striatum, although only traces of these compounds were detected in this cerebral structure from older animals. These data indicate that the

immature barrier is much more permeable to these organic acids and that treatment for HL deficient patients should be better controlled in early stages of development.

In summary, our present data clearly demonstrate that HMG and MGA disturb cell redox homeostasis in the striatum. We cannot attribute our findings to derivatives of HMG and MGA since their concentrations did not change when these organic acids were incubated with brain slices or homogenates. This is in accordance with recent findings showing that HL is not expressed in the mitochondrial fraction of the brain [35], reinforcing our results and indicating that HMG and MGA are not produced *in situ* in the brain.

In conclusion, to the best of our knowledge this is the first report showing that HMG and MGA induce *in vivo* oxidative stress in the striatum at least partially mediated by NMDA receptors. The present *in vivo* data allied to previous *in vitro* studies [16–19] indicate that this pathomechanism may potentially contribute to the neurologic manifestations found in HL deficiency. In this regard, antioxidants directed towards the mitochondria or specific antagonists of NMDA glutamate subunits focused to selectively block the toxic effects of NMDA stimulation without interfering with its physiological function in neurotransmission may possibly serve in the future as adjuvant therapies in HL deficiency, especially during crises [52,53]. In this context, it was recently shown that the NMDA receptor antagonist ketamine significantly reduced seizures and ameliorated the clinical status of a patient with nonketotic hyperglycinemia [53]. It is also possible that memantine, an open-ion channel antagonist with rapid turnover, that was shown to protect against excitotoxicity in Alzheimer's disease patients, could be used to prevent brain abnormalities in this disorder [54].

Acknowledgments

This work was supported by grants from CNPq, PRONEX II, FAPERGS, PROPESQ/UFRGS and FINEP research grant Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00, and Instituto Nacional de Ciência e Tecnologia – Excitotoxicidade e Neuroproteção (INCT-EN).

References

[1] L. Bonafé, H. Troxler, T. Kuster, C.W. Heizmann, N.A. Chamoles, A.B. Burlina, N. Blau, Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic acidurias, Mol. Genet. Metab. 69 (2000) 302–311.
[2] L. Sweetman, J.C. Williams, Branched chain organic acidurias, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, Eighth ed., McGraw-Hill, New York, 2001, pp. 2340–2342.
[3] J. Pié, E. López-Viñas, B. Puisac, S. Menao, A. Pie, C. Casale, F.J. Ramos, F.G. Hegardt, P. Gómez-Puertas, N. Casals, Molecular genetics of HMG-CoA lyase deficiency, Mol. Genet. Metab. 92 (2007) 198–229.
[4] K.M. Gibson, S.B. Cassidy, L.H. Seaver, R.J. Wanders, G.A. Mitchell, R.P. Spark, Fatal cardiomyopathy associated with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency, J. Inherit. Metab. Dis. 17 (1994) 291–294.
[5] S. Funghini, E. Pasquini, M. Cappellini, M.A. Donati, A. Morrone, C. Fonda, E. Zammarchi, 3-Hydroxy-3-methylglutaric aciduria in an Italian patient is caused by a new nonsense mutation in the HMGDL gene, Mol. Genet. Metab. 73 (2001) 268–275.
[6] C.R. Vargas, A. Sitta, G. Schmitt, G.C. Ferreira, M.L. Cardoso, D. Coelho, K.M. Gibson, M. Wajner, Incidence of 3-hydroxy-3-methylglutaryl-CoA coenzyme A lyase (HL) deficiency in Brazil, South America, J. Inherit. Metab. Dis. (2007), (SR #093).
[7] D.I. Zafeiriou, E. Vargiami, E. Mayapetek, P. Augoustidou-Sawopoulou, G.A. Mitchell, 3-Hydroxy-3-methylglutaryl coenzyme a lyase deficiency with reversible white matter changes after treatment, Pediatr. Neurol. 37 (2007) 47–50.
[8] G.N. Thompson, R.A. Chalmers, D. Halliday, The contribution of protein catabolism to metabolic decompensation in 3-hydroxy-3-methylglutaric aciduria, Eur. J. Pediatr. 149 (1990) 346–350.
[9] J. Pié, N. Casals, B. Puisac, F.G. Hegardt, Molecular basis of 3-hydroxy-3-methylglutaric aciduria, J. Physiol. Biochem. 59 (2003) 311–321.
[10] M.S. van der Knaap, H.D. Bakker, J. Valk, MR imaging and proton spectroscopy in 3-hydroxy-3-methylglutaryl coenzyme A lyase deficiency, Am. J. Neuroradiol. 19 (1998) 378–382.
[11] C. Yalçinkaya, A. Dinçer, E. Gündüz, C. Fiçicioğlu, N. Koçer, A. Aydin, MRI and MRS in HMG-CoA lyase deficiency, Pediatr. Neurol. 20 (1999) 375–380.
[12] Y. Yýlmaz, N. Ozdemir, G. Ekinci, T. Bayakal, C. Kocaman, Corticospinal tract involvement in a patient with 3-HMG coenzyme A lyase deficiency, Pediatr. Neurol. 35 (2006) 139–141.
[13] S.G. Kahler, W.G. Sherwood, D. Woof, S.T. Lawless, A. Zaritsky, J. Bonham, C.J. Taylor, J.T. Clarke, P. Durie, J.V. Leonard, Pencreatitis in patients with organic acidemias, J. Pediatr. 124 (1994) 239–243.
[14] G.A. Mitchell, T.J.C. Fukao, Inborn errors of ketone body metabolism, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, Eighth ed., McGraw-Hill, New York, 2001, pp. 2340–2342.
[15] A.A. Leung, A.K. Chan, J.A. Ezekowitz, A.K. Leung, A case of dilated cardiomiopathy associated with 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG CoA) lyase deficiency, Case Rep. Med. 183125 (2009).
[16] G. Leipnitz, B. Seminotti, J. Haubrich, K.B. Dalcin, A. Solano, G. de Bortoli, R.B. Rosa, A.U. Amaral, C.S. Dutra-Filho, A. Latini, M. Wajner, Evidence that 3-hydroxy-3-methylglutaric acid promotes lipid and protein oxidative damage and reduces the nonenzymatic antioxidant defenses in rat cerebral cortex, J. Neurosci. Res. 86 (2008) 683–693.
[17] G. Leipnitz, B. Seminotti, A.U. Amaral, G. de Bortoli, A. Solano, P.F. Schuck, A.T. Wyse, C.M. Wannmacher, A. Latini, M. Wajner, Induction of oxidative stress by the metabolites accumulating in 3-methylglutaconic aciduria in cerebral cortex of young rats, Life Sci. 82 (2008) 652–662.
[18] G. Leipnitz, B. Seminotti, C.G. Fernandes, A.U. Amaral, A.P. Beskow, L. de B. da Silva, A. Zanatta, C.A. Ribeiro, C.R. Vargas, M. Wajner, Striatum is more vulnerable to oxidative damage induced by the metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency as compared to liver, Int. J. Dev. Neurosci. 27 (2009) 351–356.
[19] C.A. Ribeiro, F.H. Hickmann, M. Wajner, Neurochemical evidence that 3-methylglutaric acid inhibits synaptic Na+, K+-ATPase activity probably through oxidative damage in brain córtex of young rats, Int. J. Dev. Neurosci. 29 (2011) 1–7.
[20] G. Paxinos, C. Watson, The rat brain in stereotaxic coordinates, Academic Press, San Diego, 1986.
[21] P. Evelson, M. Travacio, M. Repetto, J. Escobar, S. Llesuy, E.A. Lissi, Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols, Arch. Biochem. Biophys. 388 (2001) 261–266.
[22] L. Sweetman, Organic acid analysis, in: F.A. Hommes (Ed.), Techniques in Diagnostic Human Biochemical Genetics: A Laboratory Manual, Wiley-Liss, New York, 1991, p. 143.
[23] M. Wajner, D. de M. Coelho, R. Ingrassia, A.B. de Oliveira, E.N. Busanello, K. Raymond, R. Flores Pires, C.F. de Souza, R. Giugliani, C.R. Vargas, Selective screening for organic acidemias by urine organic acid GC–MS analysis in Brazil: fifteen-year experience, Clin. Chim. Acta 400 (2009) 77–81.
[24] K. Yagi, Simple procedure for specific assay of lipid hydroperoxides in serum or plasma, Methods Mol. Biol. 108 (1998) 107–110.
[25] A.Z. Reznick, L. Packer, Oxidative damage to proteins: spectrophotometric method for carbonyl assay, Methods Enzymol. 233 (1994) 357–363.
[26] C.P. LeBel, H. Ischiropoulos, S.C. Bondy, Evaluation of the probe 2′,7′-dichloroflurescin as an indicator of reactive oxygen species formation and oxidative stress, Chem. Res. Toxicol. 5 (1992) 347–352.
[27] J.A. Navarro-Gonzálvez, C. García-Benayas, J. Arenas, Semiautomated measurement of nitrate in biological fluids, Clin. Chem. 44 (1998) 679–681.
[28] R.W. Browne, D. Armstrong, Reduced glutathione and glutathione disulfide, Methods Mol. Biol. 108 (1998) 347–352.
[29] S.L. Marklund, Pyrogallol autoxidation, Handbook for Oxygen Radical Research, CRC Press, Boca Raton, FL, 1985, pp. 243–247.
[30] I. Carlberg, B. Mannervik, Glutathione reductase, Methods Enzymol. 113 (1985) 484–490.
[31] S.F. Leong, J.B. Clark, Regional development of glutamate dehydrogenase in the rat brain, J. Neurochem. 43 (1984) 106–111.
[32] A. Wendel, Glutathione peroxidise, Methods Enzymol. 77 (1981) 325–332.
[33] H. Aebi, Catalase *in vitro*, Methods Enzymol. 105 (1984) 121–126.
[34] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[35] B. Puisac, M. Arnedo, C.H. Casale, M.P. Ribate, T. Castiella, F.J. Ramos, A. Ribes, C. Pérez-Cerdá, N. Casals, F.G. Hegardt, J. Pié, Differential HMG-CoA lyase expression in human tissue provides clues about 3-hydroxy-3-methylglutaric aciduria, J. Inherit. Metab. Dis. 33 (2010) 405–410.
[36] R.J. Reiter, D.X. Tan, L.C. Manchester, W. Qi, Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence, Cell Biochem. Biophys. 34 (2001) 237–256.
[37] V.N. Anisimov, Premature ageing prevention: limitations and perspectives of pharmacological interventions, Curr. Drug Targets 7 (2006) 1485–1503.
[38] B. Halliwell, J.M.C. Gutteridge, Measurement of reactive species, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Fourth ed., Oxford University Press, Oxford, 2007, pp. 268–340.
[39] I. Dalle-Donne, D. Giustarini, R. Colombo, R. Rossi, A. Milzani, Protein carbonylation in human diseases, Trends Mol. Med. 9 (2003) 169–176.
[40] R.L. Levine, J.A. Williams, E.R. Stadman, E. Shacter, Carbonyl assays for determination of oxidatively modified proteins, Methods Enzymol. 233 (1994) 346–357.
[41] E. Marchesi, C. Rota, Y.C. Fann, C.F. Chignell, R.P. Mason, Photoreduction of the fluorescent dye 2′-7′-dichloroflurescein: a spin trapping and direct electron spin resonance study with implications for oxidative stress measurements, Free Radic. Biol. Med. 26 (1999) 148–161.
[42] D.C. Joshi, J.C. Bakowska, Determination of mitochondrial membrane potential and reactive oxygen species in live rat cortical neurons, J. Vis. Exp. 51 (2011) 2704.
[43] G.C. Brown, Nitric oxide and neuronal death, Nitric Oxide 23 (2010) 153–165.

[44] P. Singh, A. Jain, G. Kaur, Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage, Mol. Cell. Biochem. 260 (2004) 153–159.
[45] E.K. Hodgson, I. Fridovich, The interaction of bovine erythtocyte superoxide dismutase with hydrogen peroxide: inactivation of the enzyme, Biochemistry 14 (1975) 5294–5298.
[46] E. Pigeolet, P. Corbisier, A. Houbion, D. Lambert, C. Michiels, M. Raes, M.D. Zachary, J. Remacle, Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals, Mech. Ageing Dev. 51 (1990) 283–297.
[47] S. Kaushik, J. Kaur, Chronic cold exposure affects the antioxidant defenses system in various rat tissues, Clin. Chim. Acta 333 (2003) 69–77.
[48] C. Ayata, G. Ayata, H. Hara, R.T. Matthews, M.F. Beal, R.J. Ferrante, M. Endres, A. Kim, R.H. Christie, C. Waeber, P.L. Huang, B.T. Hyman, M.A. Moskowitz, Mechanisms of reduced striatal NMDA excitotoxicity in type I nitric oxide synthase knock-out mice, J. Neurosci. 17 (1997) 6908–6917.
[49] G. Kroemer, J.C. Reed, Mitochondrial control of cell death, Nat. Med. 6 (2000) 513–519.
[50] K. Nizuma, H. Endo, P.H. Chan, Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival, J. Neurochem. 109 (2009) 133–138.
[51] M.L. Circu, T.Y. Aw, Reactive oxygen species, cellular redox systems, and apoptosis, Free Radic. Biol. Med. 48 (2010) 749–762.
[52] P. Cassina, A. Cassina, M. Pehar, R. Castellanos, M. Gandelman, A. de León, K.M. Robinson, R.P. Mason, J.S. Beckman, L. Barbeito, R. Radi, Mitochondrial dysfunction in SOD1G93A-bearing astrocytes promotes motor neuron degeneration: prevention by mitochondrial-targeted antioxidants, J. Neurosci. 28 (2008) 4115–4122.
[53] Y. Suzuki, S. Kure, M. Oota, H. Hino, M. Fukuda, Nonketotic hyperglycinemia: proposal of a diagnostic and treatment strategy, Pediatr. Neurol. 43 (2010) 221–224.
[54] D. Olivares, V.K. Deshpande, Y. Shi, D.K. Lahiri, N.H. Greig, J.T. Rogers, X. Huang, N-methyl-D-aspartate receptor antagonists and memantine treatment for Alzheimer's disease, vascular dementia and Parkinson's disease, Curr. Alzheimer Res. 9 (2012) 746–758.

Capítulo 2

Induction of a proinflammatory response in cortical astrocytes by the major metabolites accumulating in HMG-CoA lyase deficiency: the role of ERK signaling pathway in cytokine release

Carolina Gonçalves Fernandes, Marília Danyelle Nunes Rodrigues, Bianca Seminotti, Ana Laura Colín-González, Abel Santamaria, André Quincozes-Santos, Moacir Wajner

Artigo científico submetido ao periódico

Molecular Neurobiology

Molecular Neurobiology

Induction of a proinflammatory response in cortical astrocytes by the major metabolites accumulating in HMG-CoA lyase deficiency: the role of ERK signaling pathway in cytokine release

--Manuscript Draft--

Manuscript Number:	
Article Type:	Original Research
Keywords:	3-Hydrohy-3-methylglutaric aciduria; oxidative stress; proinflamatory response; astrocytes; cytokine production; ERK pathway.
Corresponding Author:	Moacir Wajner, M.D. Ph.D. Universidade Federal do Rio Grande do Sul Porto Alegre, Rio Grande do Sul BRAZIL
First Author:	Carolina Gonçalves Fernandes, MSc
Order of Authors:	Carolina Gonçalves Fernandes, MSc
	Marília Danyelle Nunes Rodrigues, PhD
	Bianca Seminotti, PhD
	Ana Laura Colín-González
	Abel Santamaría, PhD
	André Quincozes-Santos, PhD
	Moacir Wajner, M.D. Ph.D.
Abstract:	3-Hydrohy-3-methylglutaric aciduria (HMGA) is an inherited metabolic disorder caused by 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. It is biochemically characterized by predominant tissue accumulation and high urinary excretion of 3-hydroxy-3-methylglutarate (HMG) and 3-methylglutarate (MGA), as well as 3-methyglutaconate and 3-hydroxyisovalerate. Affected patients commonly present acute symptoms during metabolic decompensation, including vomiting, seizures and lethargy/coma accompanied by metabolic acidosis and hypoketotic hypoglycaemia. Although neurological manifestations are common, the pathogenesis of brain injury in this disease is poorly known. Astrocytes are important for neuronal protection and are susceptible to damage by neurotoxins. In the present study, we investigated the effects of HMG and MGA, the major metabolites accumulating in HMGA, on important parameters of redox homeostasis and cytokine production in cortical cultured astrocytes. The role of the metabolites on astrocyte mitochondrial function (thiazolyl blue tetrazolium bromide - MTT reduction) and viability (propidium iodide incorporation) was also studied. Both organic acids decreased astrocytic mitochondrial function withouth altering cell viability, and the concentrations of reduced glutathione. In contrast, they increased reactive species formation (2'-7'-dichlorofluorescein diacetate - DCFHDA oxidation). They also provoked a significant increase of IL-1, IL-6 and TNFα release through the ERK signaling pathway. Taken together, the data indicate that the principal compounds accumulating in HMGA induce a proinflammatory response in cultured astrocytes that may possibly be involved in the neuropathology of this disease.

Cover Letter
Click here to download Cover Letter: Cover letter MWajner Molecular Neurobiology.doc

Departamento de Bioquímica
Instituto de Ciências Básicas da Saúde
Universidade Federal do Rio Grande do Sul
Rua Ramiro Barcelos, 2600 Anexo
Porto Alegre – RS – Brasil
90.035-003
Fax +55 51 33085540
E-mail: mwajner@ufrgs.br

January 8, 2015

Dr. Nicolas G. Bazan,
Editor-in-Chief,
Molecular Neurobiology

Dear Dr. Bazan,

I am sending you our manuscript entitled "**Induction of a proinflammatory response in cortical astrocytes by the major metabolites accumulating in HMG-CoA lyase deficiency: the role of ERK signaling pathway in cytokine 'release",** which we would like to submit for publication in Molecular Neurobiology.

This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (Eighth edition, 2011) and was approved by the Ethical Committee of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data. The manuscript does not contain clinical studies or patient data.

I also inform you that all authors have contributed significantly to the manuscript and agree with the submission of the paper at its present version. Moreover, the whole manuscript, or parts of it, will not be submitted elsewhere for publication. Finally, there is no conflict of interest in the conduct and reporting of research.

This manuscript presents novel findings showing that 3-hydroxy-3-methylglutarate and 3-methylglutarate that accumulate in 3-hydrohy-3-methylglutaric aciduria induce a proinflammatory response in cultured astrocytes that may possibly be involved in the neuropathology of this disease.

I look forward to hearing from you in the near future.

Yours sincerely,

M. Wajner, MD, PhD

Induction of a proinflammatory response in cortical astrocytes by the major metabolites accumulating in HMG-CoA lyase deficiency: the role of ERK signaling pathway in cytokine release

Carolina Gonçalves Fernandes[1], Marília Danyelle Nunes Rodrigues[1], Bianca Seminotti[1], Ana Laura Colín-González[2], Abel Santamaria[2], André Quincozes-Santos[1], Moacir Wajner[1,3*]

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

[2]Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, SSA, Mexico City, Mexico

[3]Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

Running title: The major metabolites accumulating in HMG-CoA lyase deficiency induce proinflamatory response in astrocytes

* Corresponding Author: Moacir Wajner

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul. Rua Ramiro Barcelos N° 2600 – Anexo, CEP 90035-003, Porto Alegre, RS – Brasil. Phone: +55 51 3308-5571, fax: +55 51 3308-5535, e-mail: mwajner@ufrgs.br

Abstract

3-Hydrohy-3-methylglutaric aciduria (HMGA) is an inherited metabolic disorder caused by 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. It is biochemically characterized by predominant tissue accumulation and high urinary excretion of 3-hydroxy-3-methylglutarate (HMG) and 3-methylglutarate (MGA), as well as 3-methyglutaconate and 3-hydroxyisovalerate. Affected patients commonly present acute symptoms during metabolic decompensation, including vomiting, seizures and lethargy/coma accompanied by metabolic acidosis and hypoketotic hypoglycaemia. Although neurological manifestations are common, the pathogenesis of brain injury in this disease is poorly known. Astrocytes are important for neuronal protection and are susceptible to damage by neurotoxins. In the present study, we investigated the effects of HMG and MGA, the major metabolites accumulating in HMGA, on important parameters of redox homeostasis and cytokine production in cortical cultured astrocytes. The role of the metabolites on astrocyte mitochondrial function (thiazolyl blue tetrazolium bromide – MTT reduction) and viability (propidium iodide incorporation) was also studied. Both organic acids decreased astrocytic mitochondrial function withouth altering cell viability, and the concentrations of reduced glutathione. In contrast, they increased reactive species formation (2'-7'-dichlorofluorescein diacetate - DCFHDA oxidation). They also provoked a significant increase of IL-1β, IL-6 and TNFα release through the ERK signaling pathway. Taken together, the data indicate that the principal compounds accumulating in HMGA induce a proinflammatory response in cultured astrocytes that may possibly be involved in the neuropathology of this disease.

Kew words: 3-Hydrohy-3-methylglutaric aciduria; oxidative stress; proinflamatory response; astrocytes; cytokine production**;** ERK pathway.

Introduction

3-Hydroxy-3-methylglutariyl-CoA lyase (EC 4.1.3.4) catalyses the cleavage of 3-hydroxy-3-methylglutaryl-CoA to form acetyl-CoA and acetoacetate. This reaction is a key step in ketogenesis and is also the last step in the leucine catabolic pathway [1-2]. Deficiency in this enzyme activity is the cause of to the autosomal recessive disorder called 3-hydroxy-3-methylglutaric aciduria (HMGA, MIM 246450). Biochemically, the disease is characterized by predominant tissue accumulation and high urinary excretion of 3-hydroxy-3-methylglutarate (HMG) and 3-methylglutarate (MGA), as well as 3-methylglutaconate, 3-hydroxyisovalerate and 3-methylcrotonylglycine to a lesser degree [3-8].

Clinical presentation usually occurs in the first year of life during fasting or intercurrent illnesses, mainly infections. Acute symptoms include vomiting, seizures and lethargy/coma, accompanied by metabolic acidosis, hypoketotic hypoglycemia and mild hyperammonemia [1, 10-11, 19]. Chronically progressive brain injury and mild hepatic alterations during crises are frequently present in this disorder. Pancreas and heart can also be involved in some patients [8, 12-17]. Magnetic resonance neuroimaging frequently shows multiple and marked coalescent lesions in periventricular subcortical white matter and arcuate fibers, most prominent in frontal or periatrial regions, apart from alterations in the basal ganglia attesting the significance of the neurological involvement [17-20].

Astrocytes, the more versatile cells in the mammalian brain, provide neuronal support and stability, being therefore important modulators of normal brain functioning [21-22]. Moreover, these cells have crucial role in neurodegenerative disorders, playing decisive roles in damaging and stress responses by synthesizing cytokines and chemokines [23]. This process usually lead to reactive astrogliosis and may be beneficial or deleterious because reactive astrocytes may underlie pro- or anti-inflammatory effects, being the proinflammatory response involved in pathogenesis of neurodegenerative processes [24]. Additionally, astrocytes modulate the biosynthesis and release of antioxidant defenses like glutathione (GSH) in the CNS [25].

The pathophysiology of 3-hydroxy-3-methylglutaril-CoA lyase (HMG-CoA lyase) deficiency is only partially understood. It may be explained by energy deficit (hypoketotic hypoglycaemia), reduction of fatty acid oxidation due to carnitine deficiency or alternatively due to the intracellular accumulation of ammonia and/or the toxic organic acids accumulating secondary to the leucine catabolic pathway blockage. Previous experimental evidence supports neurotoxic effects of the major compounds that accumulate intramitochondrially in HMGA. Oxidative stress has been proposed as one of the primary causes of neurodegeneration, not only through the structural and functional alterations that reactive oxygen species (ROS) produce to cell biomolecules, but also because they are potential mediators of cell death by either necrosis or apoptosis [26]. In this context, previous in vitro and ex vivo studies demonstrated that HMG and MGA induce lipid and protein oxidative damage and reduce the non-enzymatic antioxidant defenses in cerebral cortex, striatum and liver of young rats [27-30]. It was also shown that MGA reduces mitochondrial redox potential (mitochondrial dysfunction) and inhibits Na+,K+-ATPase activity in synaptosomes prepared from rat brain [31].

However, to the best of our knowledge, nothing has been reported on the influence of these metabolites on astrocytes to establish whether these cells are vulnerable to the major metabolites accumulating in HMGA. Since cultured astrocytes have made important contributions to the understanding of

neurodegenerative pathologies because of their critical role on the neuronal-astrocytic interactions [32-33], in the present work we investigated the effects of HMG and MGA on oxidative and inflammatory parameters in cortical primary astrocyte cell cultures. To further clarify the pathogenesis of the brain damage in HMGA, we explored the putative mechanisms involved in inflammatory response induced by these organic acids in astrocytes

Material and Methods

1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and other materials for cell cultures were purchased from Gibco. DNase and polyclonal anti-GFAP was purchased from Dako, whereas thiazolyl blue tetrazolium bromide (MTT), propidium iodide (Pi) and ELISA for NFκB, TNF-α, IL-1β and IL-6 were purchased from Invitrogen, PeproTech and eBioscience, respectively. All other chemicals were obtained from Sigma–Aldrich.

2. Animals

Male Wistar rats (1 to 3 days old) were obtained from our breeding colony (Department of Biochemistry, UFRGS, Brazil) and maintained in a controlled environment (12 h light/12 h dark cycle; 22 ± 1 °C; ad libitum access to food and water). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil and followed the ''Principles of Laboratory Animal Care (NIH publication 85-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

3. Primary astrocyte cultures

Animals had their cerebral cortex asseptically removed from cerebral hemispheres. All meninges were removed. During the dissection, the cerebral cortex was kept in HBSS (Hank's Balanced Salt Solution) containing 0.05% trypsin and 0.003% DNase at 37 ºC for 8 min. The tissue was then mechanically dissociated for 7 min using a Pasteur pipette and centrifuged at 100 g for 5 min. The cells were resuspended in HBSS containing DNase (0.003%) and left for decantation during 20 min. The supernatant was collected and centrifuged for 400 g for 7 min. The cells from the supernatant were resuspended in DMEM (10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM NaHCO3, 1% fungizone and 0.04% gentamicin) and plated in 24-well plates pre-coated with poly-L-lysine at a density of 3–5 x 10^5 cells/cm^2. The cells were cultured at 37 ºC in a 95% air / 5% CO_2 incubator. The first medium exchange occurred 24 h after obtaining the culture.

4. Organic acids treatment

After cells reached confluence, the culture medium was removed and the cells were incubated in the absence or presence of HMG or MGA (0.2 to 5 mM) for 24 h at 37 ºC in a 95% air / 5% CO_2 incubator in DMEM with 1% FBS.

5. Intracellular ROS levels

Intracellular ROS production was assessed as previously described [34] using the nonfluorescent cell permeating compound 2'-7'-dichlorofluorescein diacetate (DCFHDA) on cells under basal conditions or treated with HMG or MGA. DCFHDA is hydrolyzed by intracellular esterases to dichlorofluorescein (DCFH), which is trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. Astrocytes were treated with DCFHDA (10 mM) for 30 min at 37°C. Following DCFHDA exposure, the cells were scraped into PBS supplemented with 0.2% Triton X-100. Fluorescence was measured using a plate reader (Spectra Max M5, Molecular Devices) at excitation and emission wavelengths of 485 nm and 520 nm, respectively.

6. Mitochondrial function

Mitochondrial function was accessed by thiazolyl blue tetrazolium bromide (MTT) reduction assay, in which formazan is produced (activity of mitochondrial dehydrogenases). Briefly, 0.05 mg / ml of MTT was added to the incubation medium. After 3 hours of incubation, the medium from each well was gently removed by aspiration and dimethylsulfoxide (DMSO) was added to each well followed by incubation and shaking for 5 min. The formazan product generated during the incubation was solubilized in DMSO and measured at 560 and 650 nm. Only functional cells are able to reduce MTT.

7. Reduced glutathione (GSH) content

GSH levels were assessed as previously described [35]. Astrocyte homogenates (50 mg) were diluted in 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The supernatant was assayed with o-phthaldialdehyde (1 mg/ml methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0–500 mM). GSH concentrations were calculated as nmol/mg protein.

8. IL-1β, IL-6 and TNFα measurement

IL-1β and IL-6 was carried out in an extracellular medium, using a rat IL-1β or IL-6 ELISA from eBioscience (USA), whereas the TNFα assay was carried out in the same medium, using a rat TNF-α ELISA from Peprotech (USA). The results are expressed as the percentage of control levels. To test whether HO1 and ERK signaling pathways were involved in the production of these classical cytokines, we used their inhibitors zinc proporporphyrin IX (ZnPP IX, 10 μM) and PD98059 (5 μM), respectively, for 0.5 h before the treatment with HMG and MGA.

9. Measurement of NFkB levels

The levels of NFkB p65 in the nuclear fraction, which had been isolated from lysed cells by centrifugation, were measured using an ELISA commercial kit from Invitrogen (USA). The results are expressed as percentages relative to the control levels.

10. Measurement of astrocyte viability

Cell viability was measured by propidium iodide cell incorporation using phase contrast optics. Membrane integrity was assessed by fluorescent image analysis (Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory) of propidium iodide (Pi) uptake (7.5 mM) [36] at 37 ºC in an atmosphere of 5% CO_2 / 95% air in DMEM supplemented with 5% FBS.

11. GFAP quantification by western blot analysis

To characterize the astrocyte cultures we measured the glial fibrilar astrocyte protein (GFAP) levels by western blot in the presence or absence of 5 mM HMG or 5 mM MGA. Cells were solubilized with a lysis solution containing 4 % sodium dodecyl sulfate (SDS), 2 mM EDTA and 5 mM Tris-HCl at pH 6.8. Equal amounts of protein from each sample were boiled in a sample buffer (62.5 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 5 % β-mercaptoethanol, 10 % (v/v) glycerol, 0.002 % (w/v) bromophenol blue) and submitted to electrophoresis in a 10 % (w/v) SDS-polyacrilamide gel. The separated proteins were transferred to a nitrocellulose membrane. Equal loading of each sample was confirmed with Ponceau S staining (Sigma). The membrane was incubated with polyclonal antibodies targeting GFAP (1:3000). β-actin was used as a loading control. After incubating overnight with the primary antibody at 22 ºC, the membrane was washed and incubated with peroxidase-conjugated anti-rabbit immunoglobulin (IgG) at a dilution of 1 : 1000 for 1h. The chemiluminescence signal was detected using enhanced chemiluminescence; the resulting films were scanned and the bands were quantified using the Scion Image software (Scion Corp., Frederick, MD, USA).

12. Protein assay

Protein content was measured using Lowry's method, with bovine serum albumin as a standard [37].

13. Statistical analyses

Differences among groups were statistically analyzed using one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test and in some experiments the Student *t* test for unpaired samples was used. Only significant F and *t* values were shown. All analyses were performed using the Statistical Package for Social Sciences software, version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

1. 3-Hydroxy-3-methylglutaric (HMG) and 3-methylglutaric (MGA) acids decrease mitochondrial function in cultured astrocytes

We first evaluated the mitochondrial function by the MTT reduction assay. Cultured astrocytes exposed to HMG and MGA at 5 mM concentration for 24 h significantly decreased formazan formation (HMG: Fig. 1A [$F_{(2,6)} = 22.6$; $P < 0.001$] and MGA: Fig. 1B [$F_{(2,6)} = 60.1$; $P < 0.001$], respectively). However, astrocyte viability, measured by propidium iodide incorporation, was not significantly changed by HMG and MGA for 24 h (results not shown).

2. *3-Hydroxy-3-methylglutaric (HMG) and 3-methylglutaric (MGA) acids increase reactive oxygen species (ROS) production and reduce the antioxidant defenses in cultured astrocytes*

Next, we investigated whether HMG and MGA could alter ROS production in primary astrocyte cultures from neonatal rat cerebral cortex. Fig. 2A and B show that HMG [$F_{(4,37)} = 40.9$; $P < 0.001$] and MGA [$F_{(4,37)} = 27.5$; $P < 0.001$] significantly increased DCFH oxidation in cultured astrocytes in a dose dependent manner (HMG: $\beta = 0,88$; $P < 0.001$; MGA: $\beta = 0.81$; $P < 0.001$), indicating that these organic acids induce ROS production.

We also evaluated the effect of HMG and MGA on GSH levels, the major naturally occurring brain antioxidant. Fig. 3A and B show that HMG [$F_{(4,22)} = 16.1$; $P < 0.001$] and MGA [$F_{(4,22)} = 10.8$; $P < 0.001$] significantly decreased GSH concentrations in astrocytes in a dose dependent manner (HMG: $\beta = -0.62$; $P < 0.01$; MGA: $\beta = -0.66$; $P < 0.001$), indicating a decrease of non-enzymatic antioxidant defenses.

3. 3-Hydroxy-3-methylglutaric (HMG) and 3-methylglutaric (MGA) acids induce cytokine production in cultured astrocytes

We then investigated the inflammatory response in cultured astrocytes exposed to HMG or MGA by measuring interleukin production in the extracellular medium. Our results show that both HMG and MGA induced significant increases of the levels of interleukin 1β (IL-1β, Fig. 4A [$F_{(2,9)} = 28.7$; $P < 0.001$] and Fig. 4B [$F_{(2,9)} = 29.5$; $P < 0.001$]), interleukin 6 (IL-6, Fig. 4C [$F_{(2,9)} = 35.3$; $P < 0.001$] and Fig. 4D [$F_{(2,9)} = 42.6$; $P < 0.001$]) and tumor necrosis factor α (TNFα, Fig. 5E [$F_{(2,9)} = 40.9$; $P < 0.001$] and Fig. 4F [$F_{(2,9)} = 10.4$; $P < 0.001$]) release, indicating a proinflammatory response caused by these organic acids. It can be also seen that all effects were dose dependent (IL-1β: HMG: $\beta = 0.86$; $P < 0.001$; MGA: $\beta = 0.87$; $P < 0.001$; IL-6: HMG: $\beta = 0.77$; $P < 0.01$; MGA: $\beta = 0.92$; $P < 0.001$; TNFα: HMG: $\beta = 0.87$; $P < 0.001$; MGA: $\beta = 0.76$; $P < 0.01$). In a separate set of experiments, we observed no changes in cell integrity (evaluated by PI incorporation, data not shown), implying that the increased levels of cytokines most likely resulted from secretion of these proinflamatory factors.

4. 3-Hydroxy-3-methylglutaric (HMG) and 3-methylglutaric (MGA) acids do not activate NFkB in cultured astrocytes

We also observed that NFkB levels were not altered by exposition of cultured astrocytes to HMG and MGA for 24 h (Fig. 5A and B).

5. ERK signaling pathway mediates the 3-hydroxy-3-methylglutaric (HMG) and 3-methylglutaric (MGA)-induced proinflammatory cytokines release

In an attempt to determine the mechanisms underlying the HMG and MGA-induced increase proinflammatory cytokines, we measured cytokine production (IL-1β, IL-6 and TNFα) in the presence of ERK (PD98059) and HO1 (ZnPP IX) inhibitors. Table 1 shows that ERK inhibitor totally abolished the effects of HMG and MGA on IL-1β and TNFα levels. However, HO1 inhibitor was not able to prevent the increase in cytokines release provoked by HMG and MGA (Table 2).

6. 3-Hydroxy-3-methylglutaric (HMG) and 3-methylglutaric (MGA) do not induce reactive gliosis or alter astrocyte morphology

We finally measured the glial fibrilar astrocyte protein (GFAP) levels by western blot in cultured astrocytes exposed to 5 mM HMG or 5 mM MGA. No alterations in GFAP expression levels were detected, as compared to controls, indicating that these organic acids do not induce reactive gliosis (Fig. 6). In addition, no modification of cell morphology could be observed on microscope images (results not shown). These results demonstrated that HMG and MGA did not induce reactive gliosis neither changed cell morphology.

Discussion

Patients affected by HMG-CoA deficiency, also known as 3-hydroxy-3-methylglutaric aciduria (HMGA), accumulate predominantly HMG and MGA in their tissues and body fluids. The concentrations of these metabolites dramatically increase during acute metabolic crises, which are characterized by severe metabolic acidosis, hypoketotic hypoglycaemia, mild hyperammonaemia, vomiting, hypotonia, coma, and seizures resulting in permanent neurological damage [6, 11, 17, 20]. Although the neurological symptoms are common and cerebral MRI reveals marked lesions in periventricular and/or subcortical white matter, the mechanisms underlying the brain injury in this disease are poorly known. However, previous in vitro and ex vivo studies have shown that the major metabolites accumulating in this disorder disrupt mitochondrial dysfunction and redox homeostasis in rat brain [27-30].

Astrocytes modulate ionic homeostasis, energy metabolism, antioxidant and anti-inflammatory responses, among other important CNS functions [38-43]. Furthermore, since the utilization of primary astrocyte cultures have contributed to the understanding of the role of astrocytes in physiological and pathological conditions [33], in the present study we exposed astrocytes derived from cerebral cortex of neonatal rats to equimolar concentrations of HMG and MGA to mimic the biochemical phenotype of HMGA, and evaluated redox homeostasis, cytokine production, cell mitochondrial function and viability.

Initially we observed that HMG and MGA provoked a dose dependent increase of DCFH oxidation in cultured astrocytes, an useful probe for oxidative study. Since oxidation of DCFH occurs mainly through free reactive oxygen formation and is commonly used as a probe for the total ROS production in biological systems, it is concluded that these compounds elicited reactive species generation in an intact cell system (astrocytes), corroborating with previous findings obtained with subcellular fractions from total brain that includies all neural cells [27-31]. So, the first novel finding of the present investigation is the observation that astrocytes are involved in free radical production induced by the major metabolites accumulating in HMGA.

We also found that these organic acids markedly decreased GSH concentrations in cortical cultured astrocytes. In this context, astrocytes are considered to be a major source of GSH by synthesizing and secreting this antioxidant, thus helping the maintenance of the neuronal redox state [44-45]. GSH is the most abundant antioxidant in the brain, exerting a critical role to scavenge reactive oxygen species (ROS), protect protein sulfhydryl groups in the appropriate redox state, also regulating cell death and survival pathways [46].

Since adequate levels of antioxidants are essential to protect cells against oxidative damage and an imbalance in the pro-oxidant/antioxidant homeostasis induces oxidative stress [47], it is possible that the significant reduction of GSH induced by the metabolites accumulating in HMGA may cause loss of functionality of astrocytes under HMG and MGA exposure, as observed by the significant reduction of MTT. It is emphasized that the MTT assay is commonly used to assess mitochondrial functionality, as an indicator of cell energy and metabolic status, although it is also accepted as a marker of cell viability. Since propidium iodide incorporation was not changed in cultured astrocytes exposed to HMG and MGA under the same conditions, we presume that cell viability was preserved. Noteworthy, astrocytes have been demonstrated to be vulnerable to various toxins and in particular to MPP+ and this susceptibility seems to be dependent on a pro-oxidant condition induced by MPP+, leading astrocytes to lose functionality [48-49].

We cannot establish at the present whether the reduction of GSH levels occurred due to its oxidation by free radicals because of a high production of reactive species (increased DCFH oxidation) leading to GSH consumption, or whether the reduction of GSH levels led to an imbalance of reactive species formation leading to a pro-oxidant state. It is of note that depletion of GSH takes place during stress conditions giving rise to an inflammatory response with oxidative stress that plays a critical role in astrocytic inflammatory response [50]. In addition, glial GSH depletion induces neuroinflammation and neurotoxicity with significant augment of proinflammatory cytokines [51].

In this particular, another original contribution of our work was the demonstration of elevated levels of the cytokines IL-1β, IL-6 and TNFα in cultured astrocytes exposed to HMG and MGA. TNFα and IL1-β are synthesized predominantly by microglia and astrocytes, acting in acute inflammatory responses and these cytokines are able to induce IL-6 synthesis. IL-6 is also produced in microglia, astrocytes and neurons and plays a pivotal role in a variety of CNS functions such as induction and modulation of astrocytes reactivation, pathological inflammation and neuroprotection [50-54]. Moreover, the elevation of cytokine levels probably reflects glial reactivity that, in combination with an increase in ROS production and a decrease in antioxidant content in the brain, has been reported as primary features in the pathogenesis and development of various human diseases and of neurodegeneration [55-56]. Another point to be emphasized is that in our present study astrocyte activation was observed without GFAP overexpression [57].

ERK pathway has been implicated in the regulation of glial inflammatory response following an insult/damage and is an upstream signal transduction of NFκB [51, 58]. NFκB is considered the major inflammatory mediator in the CNS, although its mechanism of activation and subsequent translocation into the nucleus are not completely understood [59-60]. In this sense, the effects of HMG and MGA on classical proinflammatory cytokines (TNFα and IL1-β) were shown to be dependent on ERK signaling pathway. Concerning to IL-6, this cytokine presents a duality of actions, degenerative and reparative and/or anti- and proinflammatory [61]. We found here that IL-6 augment was not associated with ERK, but may be alternatively due to the increase of TNFα and IL1-β, inductors of IL-6 production [62, 50]. Additionally, we explored the involvement of HO1 cascade, another upstream of NFκB, on cytokine release induced by HMG and MGA. The ZnPP IX inhibitor did not prevent cytokine generation.

The pathophysiological importance of our present data is difficult to predict because brain concentrations of HMG and MGA are not yet established in HMGA affected patients. However, since neurological symptoms appear or become worse during these catabolic crises, in which the concentrations of the accumulating metabolites dramatically increase, it is conceivable a correlation between accumulation of these metabolites and worsening of clinical features. It should be also noted that the concentrations of organic acids in general, and in particular of dicarboxylic acids (such as HMG and MGA), that accumulate in various organic acidemias are higher in the brain as compared with the blood probably because of the low efflux of these compounds from the brain, which is in accordance with the trapping theory [63-66]. Nevertheless, the main relevance of this study is that it provides solid evidence that astrocytes derived from neonatal rats are vulnerable to the toxicans HMG and MGA.

In summary, we describe for the first time a susceptibility of neonatal rat cortical astrocytes to the toxic insults of HMG and MGA, giving rise to a proinflammatory response with increased cytokine production and oxidative stress, accompanied by mitochondrial dysfunction. Our findings confirm previous results carried out in subcellular fractions and homogenates from whole brain and indicate that disruption of astrocyte redox homeostasis caused by the major organic acids accumulating in HMGA may contribute to the neuropathology of patients affected by this disease. Furthermore, our present data indicate that cultured astrocytes represent an interesting and promising biological tool to study toxicity of compounds accumulating in inherited disorders, although their main limitation lies in the fact that it does not involve the intricate relationships between neurons and glial cells. It is therefore presumed that reactive species and citokyne production contribute to the neurological dysfunction observed in this disorder, particularly during metabolic crises, when metabolite production and brain accumulation is even higher. However, we cannot rule out that hyperammonemia and energy deprivation (hypoketotic hypoglycaemia), may also be involved in the brain injury of these patients since these conditions may result in free radical formation in the CNS [58, 67-68].Overall, these observations suggest that antioxidants may potentially be useful for therapeutic purposes to protect neural cells against oxidative damaging response in HMGA affected patients.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, # 470236/2012-4), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, # 10/0031-1), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, # 2011/50400-0), Pró-Reitoria de Pesquisa/Universidade Federal do Rio Grande do Sul (PROPESQ/UFRGS, # PIBITI 18489), Rede Instituto Brasileiro de Neurociência (IBN-Net) (# 01.06.0842-00) and Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção (INCT-EN, # 573677/2008-5).

Conflict of interest

The authors declare that there are no conflicts of interest.

References

1. Gibson KM, Breuer J, Nyhan WL (1988) 3-hydroxy-3-methylglutarylcoenzyme A lyase deficiency: review of 18 reported patients. Eur J Pediatr 148:180-186. doi: 10.1007/s10545-009-1048-5

2. Reimão S, Morgado C, Almeida IT, Silva M, Corte Real H, Campos J (2009) 3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency: initial presentation in a young adult. J Inherit Metab Dis 32:S49-S52. doi: 10.1007/s10545-009-1048-5

3. Kahler SG, Sherwood WG, Woof D, Lawless ST, Zaritsky A, Bonham J, Taylor CJ, Clarke JT, Durie P, Leonard JV (1994) Pancreatitis in patients with organic acidemias. J Pediatr 124:239-243. doi: 10.1016/S0022-3476(94)70311-6

4. Mitchell GA, Jakobs C, Gibson KM, Robert MF, Burlina A, Dionisi-Vici C, Dallaire L (1995) Molecular prenatal diagnosis of 3-hydroxy-3-methylglutaryl-coenzime A lyase deficiency. Prenat Diagn 15:725-729. doi: 10.1002/pd.1970150807

5. Bonafé L, Troxler H, Kuster T, Heizmann CW, Chamoles NA, Burlina AB, Blau N (2000) Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic acidurias. Mol Genet Metab 69:302-311. doi: 10.1006/mgme.2000.2982

6. Sweetman L, Williams JC (2001) Branched chain organic acidurias. in: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill, New York, pp 2340–2342.

7. Pié J, López-Viñas E, Puisac B, Menao S, Pie A, Casale C, Ramos FJ, Hegardt FG, Gómez-Puertas P, Casals N (2007) Molecular genetics of HMG-CoA lyase deficiency. J Inherit Metab 92:198-229. doi: 10.1016/j.ymgme.2007.06.020

8. Leung AA, Chan AK, Ezekowitz JA, Leung AK (2009) A case of dilated cardiomiopathy associated with 3-hydroxy-3methylglutaryl-Coenzyme A (HMG CoA) lyase deficiency. Case Rep Med 2009:183125. doi: 10.1155/2009/183125

9. Schutgens RB, Heymans H, Ketel A, Veder HA, Duran M, Ketting D, Wadman SK (1979) Lethal hypoglycemia in a child with a deficiency of 3-hydroxy-3-methylglutarylcoenzyme A lyase. J Pediatr 94:89-91. doi: 10.1016/S0022-3476(79)80364-9

10. Wysocki SJ, Hähnel R (1986) 3-Hydroxy-3-methylglutaryl-coenzyme a lyase deficiency: a review. J Inherit Metab Dis 9:225-233. doi: 10.1007/BF01799652

11. Pospísilová E, Mrázová L, Hrdá J, Martincová O, Zeman J (2003) Biochemical and molecular analyses in three patients with 3-hydroxy-3-methylglutaric aciduria. J inherit Metab Dis 26:433-441. doi: 10.1023/A:1025169210121

12. Wilson WG, Cass MB, Sovik O, Gibson KM, Sweetman L (1984) A child with acute pancreatitis and recurrent hypoglycemia due to 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. Eur J Pediatr 142:289-291. doi: 10.1007/B00540255

13. Zoghbi H, Spence E, Beaudet A, O´Brien WE, Goodman CJ, Gibson KM (1986) Atypical presentation and neuropathological studies in 3-hydroxy-3-methylglutaryl conenzyme A lyase deficiency. Ann Neurol 20:367-369. doi: 10.1002/ana.410200318

14. Gibson KM, Cassidy SB, Seaver LH, Wanders RJ, Mitchell GA, Spark RP (1994) Fatal cardiomyopathy associated with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. J Inherit Metab Dis 17:291-294. doi: 10.1007/BF00711810

15. Muroi J, Yorifuji T, Uematsu A, Nakahata T (2000) Cerebral infarction and pancreatitis: possible complications of patients with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. J Inherit Metab Dis 23:636-637. doi: 10.1023/A:1005642316174

16. Urgançi N, Arapoglu M, Evrüke M Aydin A (2001) A rare cause of hepatomegaly: 3-hydroxy-3-methylglutaryl coenzyme-a lyase deficiency. J Pediatr Gastroenterol Nutr 33:339-341. doi: 00005176-200109000-00022

17. Zafeiriou DI, Vargiami E, Mayapetek E, Augoustidou-Sawopoulou P, Mitchell GA (2007) 3-Hydroxy-3-methylglutaryl coenzyme a lyase deficiency with reversible white matter changes after treatment. Pediatr Neurol 37:47-50. doi: 10.1016/j.pediatrneurol.2007.02.007

18. van der Knaap MS, Bakker HD, Valk J (1998) MR imaging and proton spectroscopy in 3-hydroxy-3-methylglutaryl coenzyme A lyase deficiency. Am J Neuroradiol 19:378-382.

19. Yalçinkaya C, Dinçer A, Gündüz E, Fiçicioglu C, Koçer N, Aydin A (1999) MRI and MRS in HMG-CoA lyase deficiency. Pediatr Neurol 20:375-380. doi: 10.1016/S0887-8994(99)00013-2

20. Yýlmaz Y, Ozdemir N, Ekinci G, Bayaka T, Kocaman C (2006) Corticospinal tract involvement in a patient with 3-HMG coenzyme A lyase deficiency. Pediatr Neurol 35:139-141. doi: 10.1016/j.pediatrneurol.2006.01.009

21. Kahlert S, Reiser G (2004) Glial perspectives of metabolic states during cerebral hypoxia-calcium regulation and metabolic energy. Cell Calcium 36:295-302. doi: 10.1016/j.ceca.2004.02.009

22. Takuma K, Baba A, Matsuda T (2004) Astrocyte apoptosis: implications for neuroprotection. Prog Neurobiol 72:111-127. doi: 10.1016/j.pneurobio.2004.02.001

23. Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. Acta Neuropathol 119:7-35. doi: 10.1007/s00401-009-0619-8

24. Zhang D, Hu X, Qian L, O'Callaghan JP, Hong JS (2010) Astrogliosis in CNS pathologies: is there a role for microglia? Mol Neurobiol 41:232-241. doi: 10.1007/s12035-010-8098-4

25. Hertz L, Zielke HR (2004) Astrocytic control of glutamatergic activity: astrocytes as stars of the show. Trends Neurosci 27:735-743. doi: 10.1016/j.tins.2004.10.008

26. Friedlander RM (2003) Apoptosis and caspases in neurodegenerative diseases. N Engl J Med 348:1365-1375. doi: 10.1056/NEJMra022366

27. Leipnitz G, Seminotti B, Haubrich J, Dalcin KB, Solano A, de Bortoli G, Rosa RB, Amaral AU, Dutra-Filho CS, Latini A, Wajner M (2008a) Evidence that 3-hydroxy-3-methylglutaric acid promotes lipid and protein oxidative damage and reduces the nonenzymatic antioxidant defenses in rat cerebral cortex. J Neurosci Res 86:683-693. doi: 10.1002/jnr.21527

28. Leipnitz G, Seminotti B, Amaral AU, de Bortoli G, Solano A, Schuck PF, Wyse AT, Wannmacher CM, Latini A, Wajner M (2008b) Induction of oxidative stress by the metabolites accumulating in 3-methylglutaconic aciduria in cerebral cortex of young rats. Life Sci 82:652-662. doi: 10.1016/j.lfs.2007.12.024

29. Leipnitz G, Seminotti B, Fernandes CG, Amaral AU, Beskow AP, da Silva Lde B, Zanatta A, Ribeiro CA, Vargas CR, Wajner M (2009) Striatum is more vulnerable to oxidative damage induced by the metabolites accumulating in 3-hydroxy-4-methylglutaryl-CoA lyase deficiency as compared to liver. Int J Dev Neurosci 27:351-356. doi:10.1016/j.ijdevneu.2009.03.001

30. Fernandes CG, da Rosa MS, Seminotti B, Pierozan P, Martell RW, Lagranha VL, Busanello ENB, Leipnitz G, Wajner M (2013) In vivo experimental evidence that the major metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency induce oxidative stress in striatum of developing rats: A potential pathophysiological mechanism of striatal damage in this disorder. Mol Genet Metab 109:144-153. doi: 10.1016/j.ymgme.2013.03.017

31. Ribeiro CA, Hickmann FH, Wajner M (2011) Neurochemical evidence that 3-methylglutaric acid inhibits synaptic Na+,K+-ATPase activity probably through oxidative damage in brain cortex of young rats. Int J Dev Neurosci 29:1-7. doi: 10.1016/j.ijdevneu.2010.10.007

32. Hertz L, Peng L, Lai JC (1998) Functional studies in cultured astrocytes. Methods 16:293-310. doi: 10.1006/meth.1998.0686

33. Lange SC, Bak LK, Waagepetersen HS, Schousboe A, Norenberg, MD (2012) Primary cultures of astrocytes: their value in understanding astrocytes in health and disease. Neurochem Res 37:2569-2588. doi: 10.1007/s11064-012-0868-0

34. Quincozes-Santos A, Nardin P, de Souza DF, Gelain DP, Moreira JC, Latini A, Gonçalves CA, Gottfried C (2009) The janus face of resveratrol in astroglial cells. Neurotox Res 16, 30-41. doi: 10.1007/s12640-009-9042-0

35. Souza DG, Bellaver B, Souza DO, Quincozes-Santos A (2013) Characterization of adult rat astrocyte cultures. PLoS One 8:e60282. doi: 10.1371/journal.pone.0060282

36. dos Santos AQ, Nardin P, Funchal C, de Almeida LM, Jacques-Silva MC, Wofchuk ST, Gonçalves CA, Gottfried C (2006) Resveratrol increases glutamate uptake and glutamine synthetase activity in C6 glioma cells. Arch Biochem Biophys 453:161-167. doi: 10.1016/j.abb.2006.06.025

37. Lowry OH, Rosebrough NJ, Farr AL, Randal RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275.

38. Anderson CM, Swanson RA (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32:1-14. doi: 10.1002/1098-1136(200010)32:1<1::AID-GLIA10>3.0.CO;2-W

39. Nedergaard M, Ransom B, Goldman SA (2003) New roles for astrocytes: redefining the functional architecture of the brain. Trends Neurosci 26:523-530. doi: 10.1016/j.tins.2003.08.008

40. Wang DD, Bordey A (2008) The astrocyte odyssey. Prog Neurobiol 86:342-367. doi: 10.1016/j.pneurobio.2008.09.015

41. Bélanger M, Allaman I, Magistretti PJ (2011) Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. Cell Metab 14:724-738. doi: 10.1016/j.cmet.2011.08.016

42. Purpura KA, Bratt-Leal AM, Hammersmith KA, McDevitt TC, Zandstra PW (2012) Systematic engineering of 3D pluripotent stem cell niches to guide blood development. Biomaterials 33:1271-1280. doi: 10.1016/j.biomaterials.2011.10.051

43. Ransom BR, Ransom CB (2012) Astrocytes: multitalented stars of the central nervous system. Methods Mol Biol 814:3-7. doi: 10.1007/978-1-61779-452-0_1

44. Dringen R (2000) Metabolism and functions of glutathione in brain. Prog Neurobiol. 62**:**649-71. doi: 10.1016/S0301-0082(99)00060-X

45. Pope SA, Milton R, Heales SJ (2008) Astrocytes protect against copper-catalysed loss of extracellular glutathione. Neurochem Res 33:1410-1418. doi: 10.1007/s11064-008-9602-3

46. Sarafian TA, Bredesen DE, Verity MA (1996) Cellular resistence to methylmercury. Neurotoxicology 17:27-36.

47. Castoldi AF, Coccini T, Manzo L (2001) Biological markers of neurotoxic diseases. Funct Neurol 16:39-44.

48. Tsai MJ, Lee EH (1994) Differences in the disposition and toxicity of 10-methyl-4-phenylpyridinium in cultured rat and mouse astrocytes. Glia 12:329-335. doi: 10.1002/glia.440120409

49. Alarcón-Aguilar A, González-Puertos VY, Luna-Lopéz A, López-Macay A, Morán J, Santamaría A, Königsberg M (2014) Comparing the effects of two neurotoxins in cortical astrocytes obtained from rats of different ages: involvment of oxidative damage. J Appl Toxicol 34:127-138. doi: 10.1002/jat.2841

50. Tanabe K, Matsushima-Nishiwaki R, Yamaguchi S, Iida H, Dohi S, Kozawa O (2010) Mechanisms of tumor necrosis factor-alpha-induced interleukin-6 synthesis in glioma cells. J Neuroinflammation 7:16. doi: 10.1186/1742-2094-7-16

51. Lee M, Cho T, Jantaratnotai N, Wang YT, McGeer E, McGeer PL (2010) Depletion of GSH in glial cells induces neurotoxicity: relevance to aging and degenerative neurological diseases. FASEB J 24:2533-2545. doi: 10.1096/fj.09-149997

52. Santello M, Bezzi P, Volterra A (2011) TNFα controls glutamatergic gliotransmission in the hippocampal dentate gyrus. Neuron 69:988-1001. doi: 10.1016/j.neuron.2011.02.003

53. Erta M, Quintana A, Hidalgo J (2012) Interleukin-6, a major cytokine in the central nervous system. Int J Biol Sci 8:1254-1266. doi: 10.7150/ijbs.4679

54. Ye L, Huang Y, Zhao L, Li Y, Sun L, Zhou Y, Qian G, Zheng JC (2013) IL-1β and TNF-α induce neurotoxicity through glutamate production: a potential role for neuronal glutaminase. J Neurochem 125:897-908. doi: 10.1111/jnc.12263

55. Darlington CL (2005) Astrocytes as targets for neuroprotective drugs. Curr Opin Investig Drugs 6:700-703.

56. Kanwar SS, Nehru B (2007) Modulatory effects of N-acetylcysteine on cerebral cortex and cerebellum regions of ageing rat brain. Nutr Hosp 22:95-100.

57. Brenner M (2014) Role of GFAP in CNS injuries. Neurosci Lett 565:7-13. doi: 10.1016/j.neulet.2014.01.055

58. Bobermin LD, Quincozes-Santos A, Guerra MC, Leite MC, Souza DO, Gonçalves CA, Gottfried C (2012) Resveratrol prevents ammonia toxicity in astroglial cells. PLoS One 7:e52164. doi: 10.1371/journal.pone.0052164

59. Gloire G, Legrand-Poels S, Piette J (2006). NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 72:1493-1505. doi: 10.1016/j.bcp.2006.04.011

60. Wakabayashi N, Slocum SL, Skoko JJ, Shin S, Kensler TW (2010) When NRF2 talks, who's listening? Antioxid Redox Signal 13:1649-1663. doi: 10.1089/ars.2010.3216

61. Farina C, Aloisi F, Meinl E (2007) Astrocytes are active players in cerebral innate immunity. Trends Immunol 28:138-145. doi: 10.1016/j.it.2007.01.005

62. Tanabe K, Kozawa O, Iida H (2011) Midazolam suppresses interleukin-1beta-induced interleukin-6 release from rat glial cells. J Neuroinflammation 8:68. doi: 10.1186/1742-2094-8-68.

63. Hoffmann GF, Meier-Augenstein W, Stockler S, Surtees R, Rating D, Nyhan WL (1993) Physiology and pathophysiology of organic acids in cerebrospinal fluid. J Inherit Metab Dis 16:648-669. doi: 10.1007/BF00711898

64. Hoffmann GF, Gibson KM, Trefz FK, Nyhan WL, Bremer HJ, Rating D (1994) Neurological manifestations of organic acid disorders. Eur J Pediatr 153:S94-S100. doi: 10.1007/BF02138774

65. Sauer SW, Okun JG, Fricker G, Mahringer A, Müller I, Crnic LR, Mühlhausen C, Hoffmann GF, Hörster F, Goodman SI, Harding CO, Koeller DM, Kölker S (2006) Intracerebral accumulation of glutaric and 3-hydroxyglutaric acids secondary to limited flux across the blood-brain barrier constitutes a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. J Neurochem 97:899-910. doi: 10.1111/j.1471-4159.2006.03813.x

66. Stellmer F, Keyser B, Burckhardt BC, Koepsell H, Streichert T, Glatzel M, Jabs S, Thiem J, Herdering W, Koeller DM, Goodman SI, Lukacs Z, Ullrich K, Burckhardt G, Braulke T, Mühlhausen C (2007) 3-Hydroxyglutaric acid is transported via the sodium-dependent dicarboxylate transporter NaDC3. J Mol Med (Berl) 85:763-770. doi: 10.1007/s00109-007-0174-5

67. Singh P, Jain A, Kaur G (2004) Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage. Mol Cell Biochem 260:153-159. doi: 10.1023/B:MCBI.0000026067.08356.13

68. Suh SW, Gum ET, Hamby AM, Chan PH, Swanson RA (2007) Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. J Clin Invest 117:910-918. doi: 10.1172/JCI30077

Legend to figures

Fig. 1 *In vitro* effects of 3-hydroxy-3-methylglutaric (HMG) and 3-methylglutaric acids (MGA) on thiazolyl blue tetrazolium bromide (MTT) reduction in astrocyte cultures from cerebral cortex of rats. Data are represented as mean ± SD of 3 independent experiments (wells) performed in triplicate and expressed as percentage of controls ** $P < 0.01$, *** $P < 0.001$, compared to control (Duncan´s multiple range test)

Fig. 2 *In vitro* effects of 3-hydroxy-3-methylglutaric (HMG, A) and 3-methylglutaric acids (MGA, B) on 2',7'-dichlorofluorescein diacetate (DCFH-DA) oxidation in astrocyte cultures from cerebral cortex of rats. Data are represented as mean ± SD of 7 to 10 independent experiments (wells) performed in triplicate and expressed as percentage of controls * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to control and expressed as percentage of controls (Duncan´s multiple range test)

Fig. 3 *In vitro* effects of 3-hydroxy-3-methylglutaric (HMG, A) and 3-methylglutaric acids (MGA, B) on reduced glutathione (GSH) levels in astrocyte cultures from cerebral cortex of rats. Data are represented as mean ± SD of 5 to 6 independent experiments (wells) performed in triplicate and expressed as percentage of controls. ** $P < 0.01$, *** $P < 0.001$, compared to control (Duncan´s multiple range test)

Fig. 4 *In vitro* effects of 3-hydroxy-3-methylglutaric (HMG) and 3-methylglutaric acids (MGA) on the release of the cytokines interleukin 1β (IL-1β - A, B), interleukin 6 (IL-6 - C, D) and tumor necrosis factor α (TNF α - E, F) in astrocyte cultures from cerebral cortex of rats after 24 hours of exposition. Data are represented as mean ± SD of 4 independent experiments (wells) performed in triplicate and expressed as percentages of controls. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to control (Duncan´s multiple range test)

Fig. 5 *In vitro* effects of 3-hydroxy-3-methylglutaric (HMG) and 3-methylglutaric acids (MGA) on the NFkB levels in astrocyte cultures from cerebral cortex of rats after 24 hours of exposition. Data are represented as mean ± SD of 4 independent experiments (wells) performed in triplicate and expressed as percentages of controls. No differences between groups were detected (one-way ANOVA)

Fig. 6 *In vitro* effects of 3-hydroxy-3-methylglutaric (HMG) and 3-methylglutaric acids (MGA) on GFAP expression levels in astrocyte cultures from cerebral cortex of rats after 24 hours of exposition. Data are represented mean ± SD of 2 independent experiments (wells) performed in triplicate and expressed as percentage of controls. No differences between groups were detected (one-way ANOVA)

Table1
Click here to download Table: Table 1.doc

Table 1 - Effects of the ERK inhibitor PD 98059 (5 μM) on 3-hydroxy-3-methylglutaric (5 mM HMG) and 3-methylglutaric acids (5 mM MGA)-induced alterations on the release of the cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor α (TNF α) in astrocyte cultures from cerebral cortex of rats

	IL-1β	IL-6	TNF α
Control	100	100	100
PD 98059	98.7 ± 7.20	104 ± 8.67	98.3 ± 3.78
HMG	137 ± 10.8 ***	142 ± 12.9 ***	139 ± 5.56 ***
MGA	145 ± 7.44 ***	162 ± 14.3 ***	140 ± 5.65 ***
HMG + PD 98059	102 ± 9.01 ###	147 ± 11.5 ***	106 ± 5.40 ###
MGA + PD 98059	96.3 ± 14.5 ###	160 ± 19.8 ***	106 ± 9.45 ###

Values are mean ± standard deviation for four to six independent (wells) experiments per group expressed as percentage of control. *** $p < 0.001$ compared to control group; ### $p < 0.001$ compared to HMG and MGA (Duncan´s multiple range test).

Table2
Click here to download Table: Table 2.doc

Table 2 - Effects of the heme oxygenase 1 (HO1) inhibitor zinc protoporphyrin IX (10 μM ZnPP IX) on 3-hydroxy-3-methylglutaric (5 mM HMG) and 3-methylglutaric acids (5 mM MGA)-induced alterations on the release of the cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor α (TNF α) in astrocyte cultures from cerebral cortex of rats

	IL-1β	IL-6	TNF α
Control	100 ± 0	100 ± 0	100 ± 0
ZnPP IX	98 ± 6.29	100 ± 4.03	103 ± 2.83
HMG	145 ± 8.33 ***	160 ± 8.04 ***	136 ± 5.51 ***
MGA	149 ± 16.1 ***	172 ± 18.9 ***	142 ± 3.77 ***
HMG + ZnPP IX	140 ± 11.8 ***	152 ± 9.05 ***	134 ± 5.16 ***
MGA + ZnPP IX	155 ± 6.56 ***	177 ± 6.76 ***	143 ± 13.9 ***

Values are mean ± standard deviation for 4 to 6 independent (wells) experiments per group expressed as percentage of control. *** $p < 0.001$ compared to control group (Duncan's multiple range test).

Figure1
Click here to download high resolution image

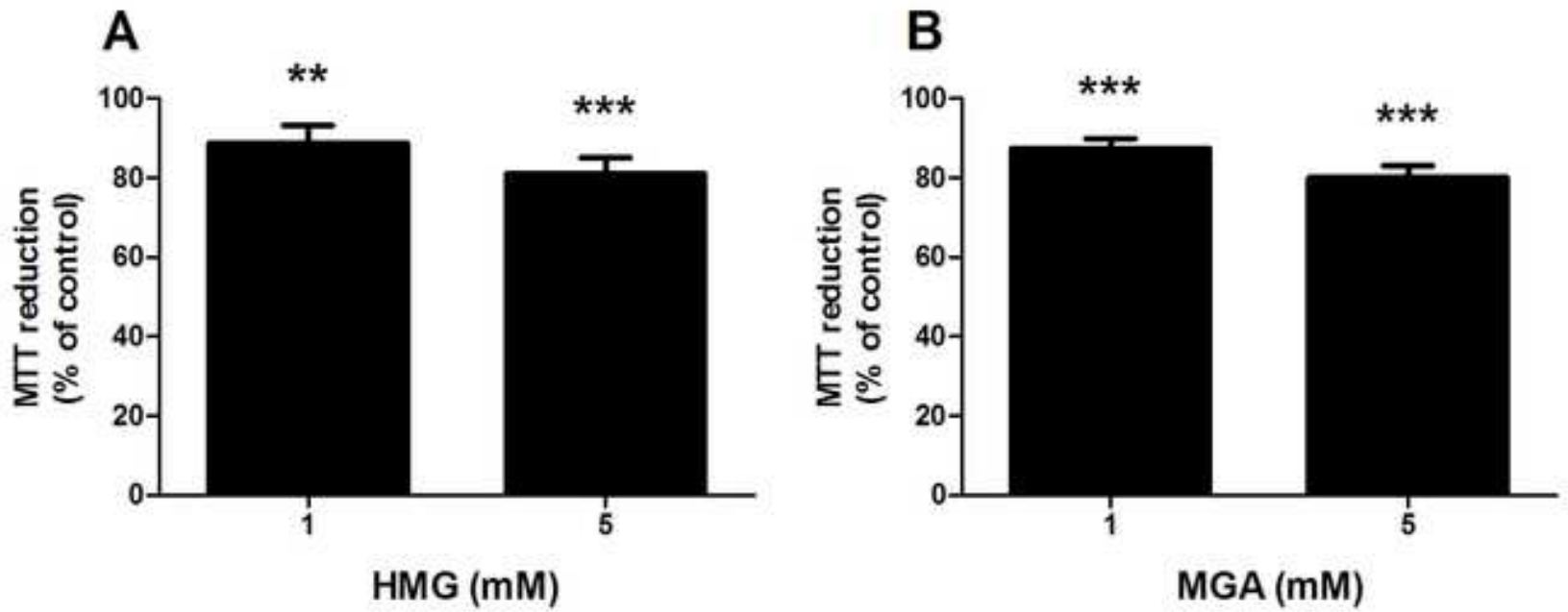


Figure2
Click here to download high resolution image

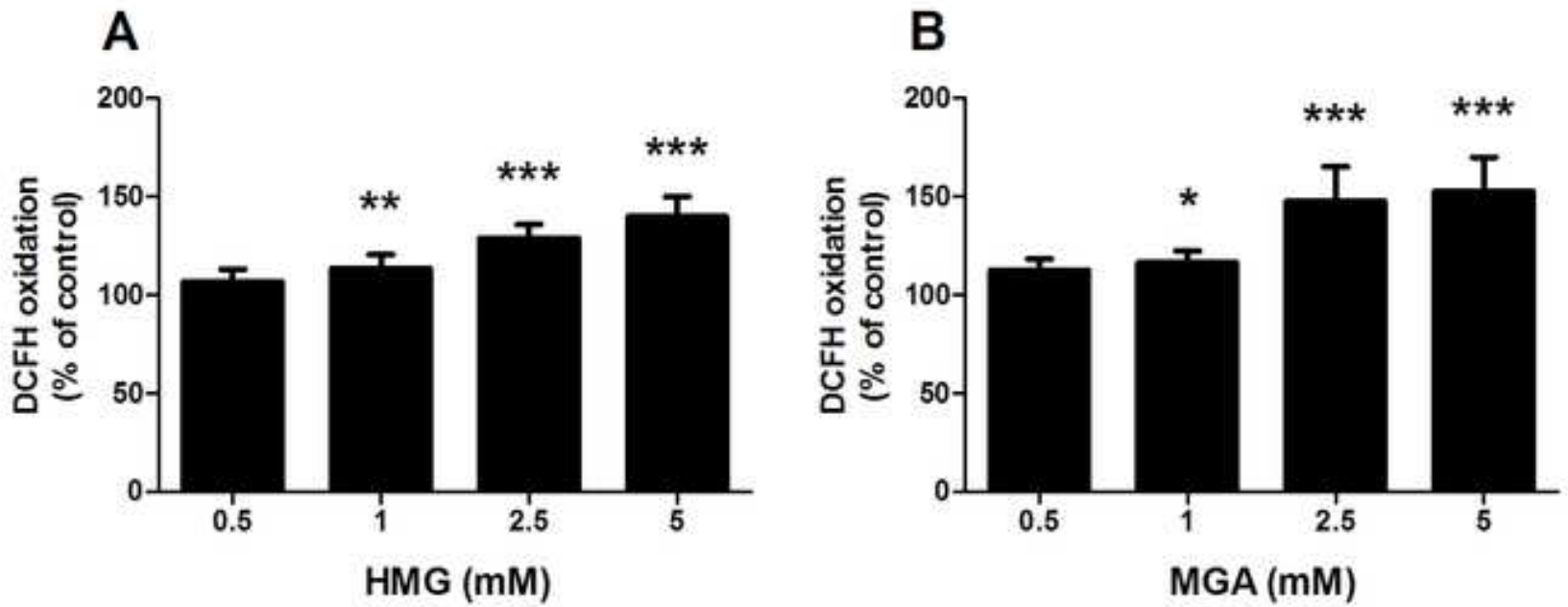


Figure3
Click here to download high resolution image

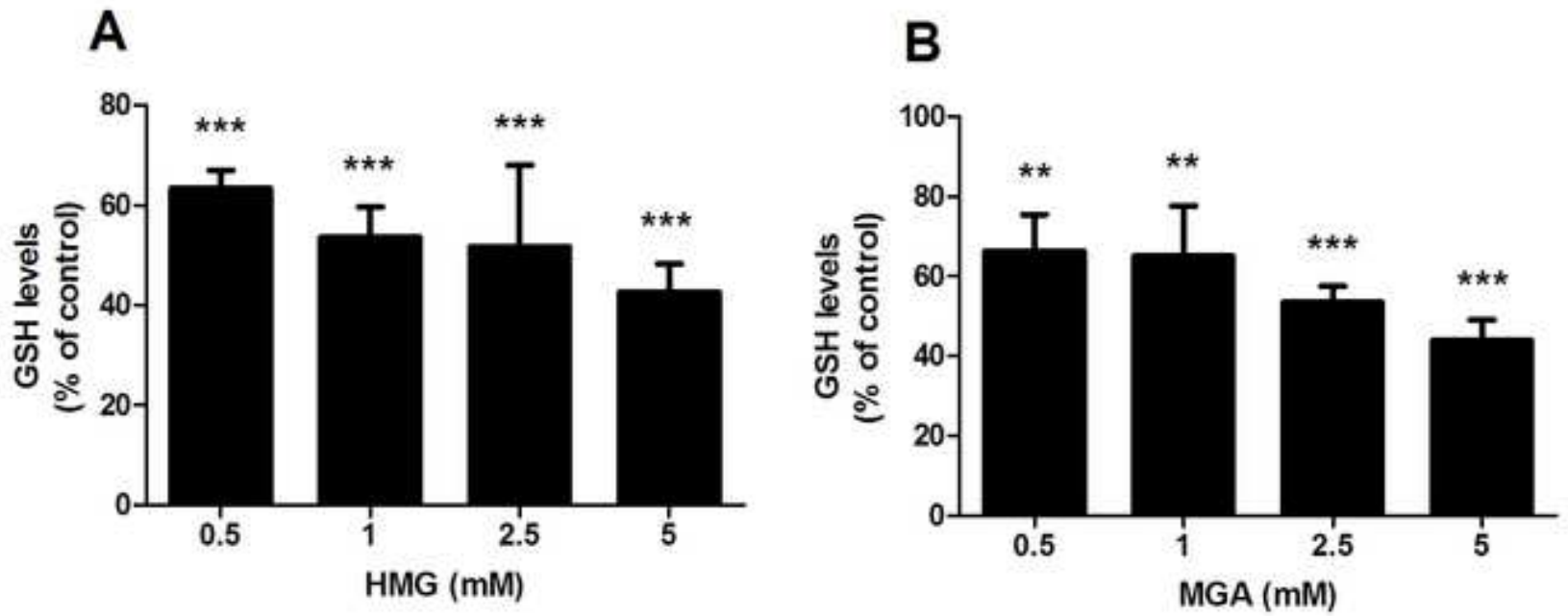


Figure4
Click here to download high resolution image

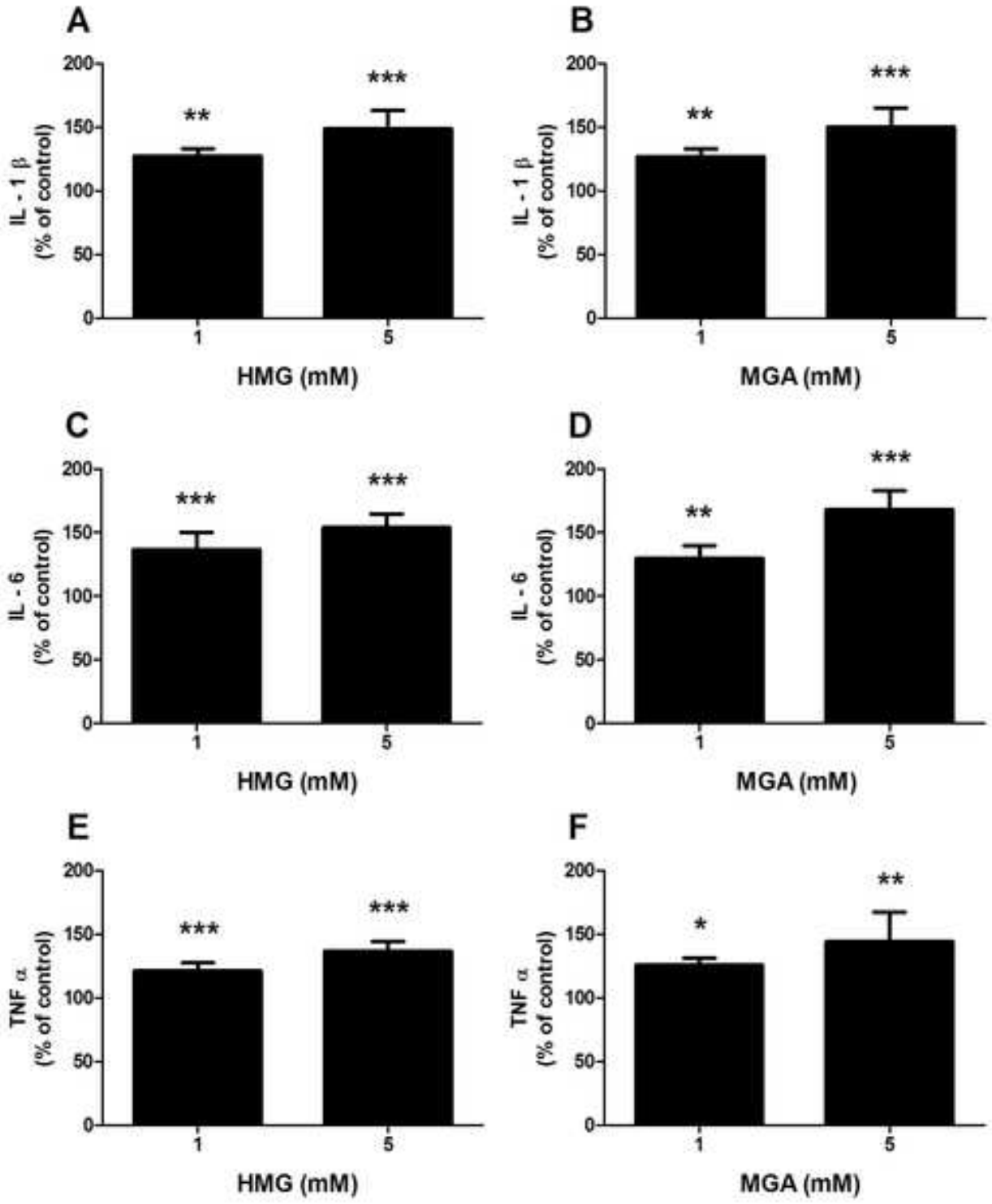


Figure5
Click here to download high resolution image

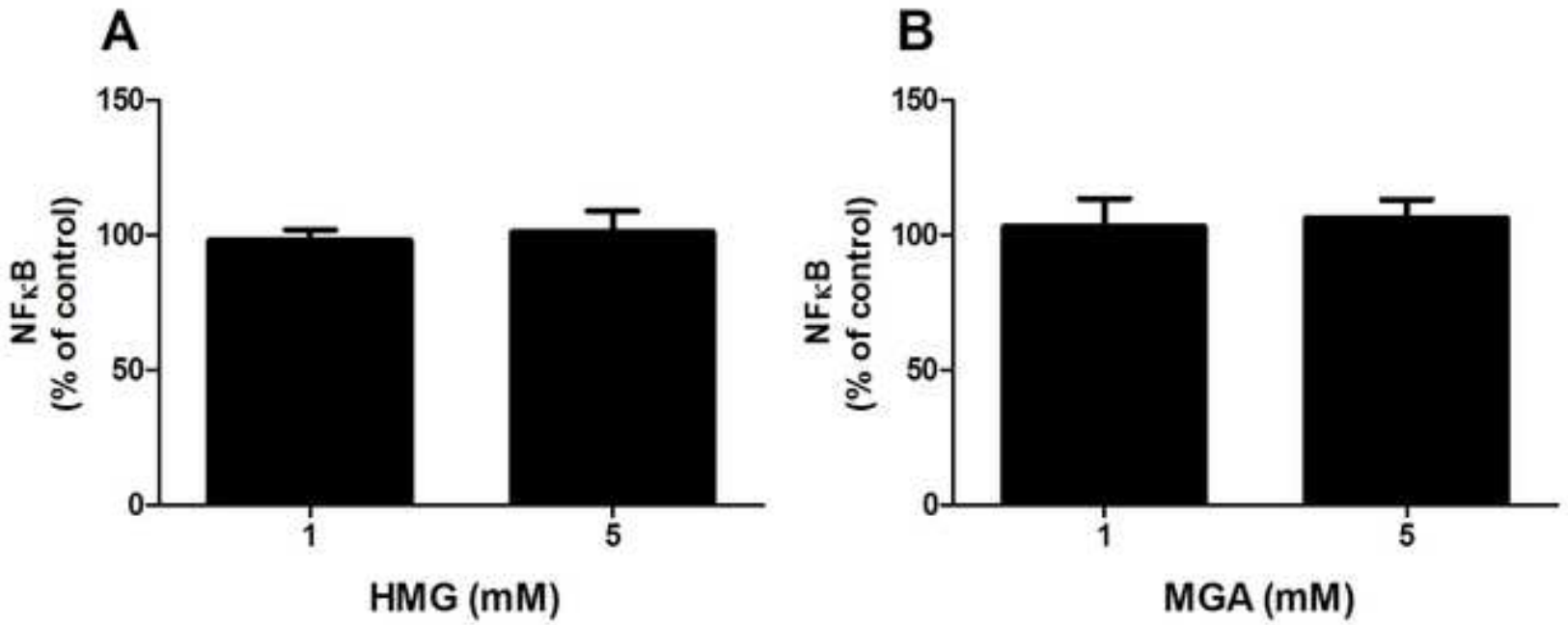
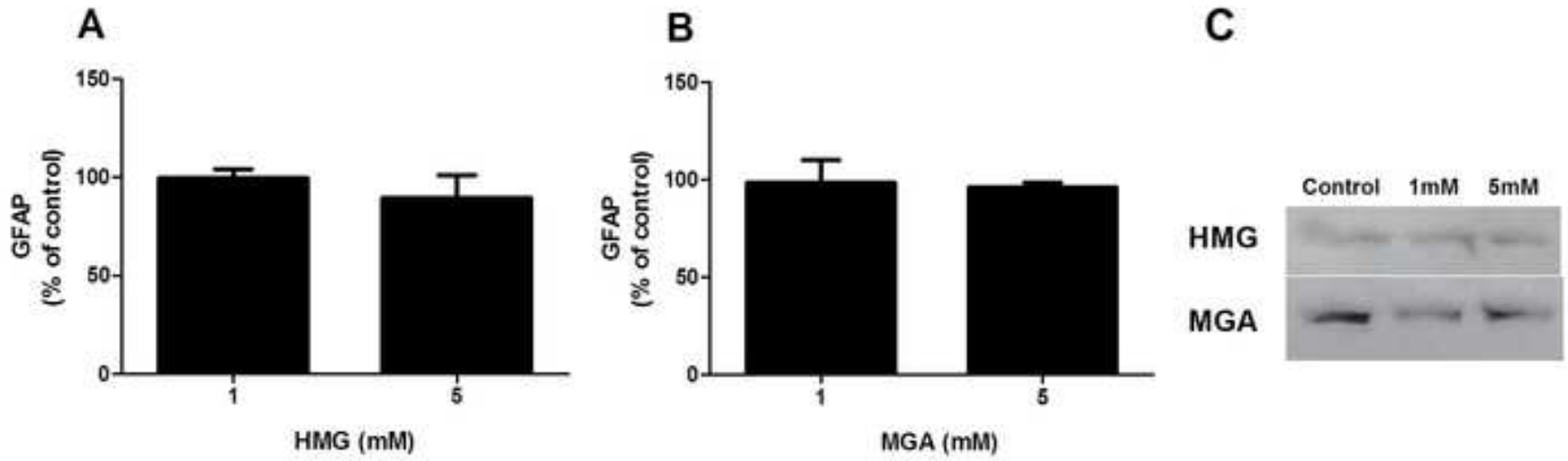


Figure6
Click here to download high resolution image



Capítulo 3

Experimental evidence that the major metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency induce hypophosphorylation of cytoskeletal proteins of neural cells mediated by protein kinase inhibition and oxidative stress

Carolina Gonçalves Fernandes, Paula Pierozan, Gilberto Machado Soares, Fernanda Ferreira, Ângela Zanatta, Alexandre Umpierrez Amaral, Clarissa Günther Borges, Moacir Wajner and Regina Pessoa-Pureur

Artigo científico submetido ao periódico

Experimental Neurology

Elsevier Editorial System(tm) for Experimental Neurology
Manuscript Draft

Manuscript Number:

Title: Experimental evidence that the major metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency induce hypophosphorylation of cytoskeletal proteins of neural cells mediated by protein kinase inhibition and oxidative stress

Article Type: Regular Paper

Section/Category: Cellular and Molecular Neuroscience

Keywords: 3-hydroxy-3-methylglutaryl-CoA lyase deficiency; hyporphosphorylation; intermediate filaments; cell signaling; glutamatergic receptors

Corresponding Author: Dr Regina Pessoa-Pureur, PhD

Corresponding Author's Institution: Universidade Federal do Rio Grande do Sul

First Author: Carolina G Fernandes, MSc

Order of Authors: Carolina G Fernandes, MSc; Paula Pierozan, PhD; Gilberto M Soares; Fernanda Ferreira; Ângela Zanatta, MSc; Alexandre U Amaral, PhD; Clarissa G Borges; Moacir Wajner, PhD; Regina Pessoa-Pureur, PhD

Abstract: 3-Hydroxy-3-methylglutaryl-CoA lyase (HL) deficiency is an inborn disease biochemically characterized by tissue accumulation and high urinary excretion of 3-hydroxy-3-methylglutaric (HMG), 3-methylglutaric (MGA), 3-methylglutaconic (MGT) acids and also of 3-hydroxyisovaleric acid to a lesser extend. Neurological symptoms and abnormalities are commonly observed in patients with this disease, but their pathogenesis is poorly known. In the present study we found that HMG, MGA and MGT caused hypophosphorylation of glial fibrillary acidic protein and the neurofilament subunits NFL, NFM and NFH in striatal and cortical slices of rats. Energy deprivation was not responsible for these effects since important parameters of mitochondrial energy production were not altered. However, HMG-induced hypophosphorylation of cytoskeletal proteins was mediated by inhibition of protein kinases, with no alterations of protein phosphatases. Thus, we demonstrated that protein cAMP-dependent kinase (PKA) inhibition was involved in the hypophosphorylation of Ser55 of NFL, as well as JNK/MAPK inhibition resulted in the hypophosphorylation of KSP repeats of NFM and NFH subunits. It was also observed that the cytoskeletal hypophosphorylation was dependent on synaptic and extra synaptic (NR2B subunit) NMDA receptors and also on Ca2+. Furthermore, the NOS inhibitor, L-NAME, and the antioxidant, TROLOX (polar analog of vitamin E), fully prevented the hypophosphorylation and the inhibition of PKA and JNK activities caused by HMG. These findings indicate that the principal metabolites accumulating in HL deficiency disrupt the cytoskeleton phosphorylating system probably through NMDA receptors, Ca2+ and disruption of redox homeostasis. It is presumed that these mechanisms may contribute to the neuropathology of this disease.

Cover Letter

Departamento de Bioquímica
Instituto de Ciências Básicas da Saúde
Universidade Federal do Rio Grande do Sul
Rua Ramiro Barcelos, 2600 Anexo
Porto Alegre – RS – Brasil
90.035-003
Fax +55 51 33085540
E-mail: rpureur@ufrgs.br

January 26, 2015

Dr. A. Hoke,
Editor-in-Chief,
Experimental Neurology

Dear Dr. Hoke,

I am sending you our manuscript entitled "**Experimental evidence that the major metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency induce hypophosphorylation of cytoskeletal proteins of neural cells mediated by protein kinase inhibition and oxidative stress",** which we would like to submit for publication in **Experimental Neurology**.

This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (Eighth edition, 2011) and was approved by the Ethical Committee of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data. The manuscript does not contain clinical studies or patient data.

I also inform you that all authors have contributed significantly to the manuscript and agree with the submission of the paper at its present version. Moreover, the whole manuscript, or parts of it, will not be submitted elsewhere for publication. Finally, there is no conflict of interest in the conduct and reporting of research.

This manuscript presents novel findings showing that 3-hydroxy-3-methylglutarate, 3-methylglutarate and 3-methylglutaconate that accumulate in 3-hydrohy-3-methylglutaric aciduria induce a disruption in the cytoskeletal-associated phosphorylating system probably through NMDA receptors, Ca^{2+} and disruption of redox homeostasis that may possibly be involved in the neuropathology of this disease.

I look forward to hearing from you in the near future.

Yours sincerely,

Pessoa-Pureur R. PhD

Suggested Reviewers

- **Abel Santamaría**

Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirurgía, SSA

e-mail: absada@yahoo.com

- **Magdalena Ugarte**

Dept. Biologia Molecular, Universidade Autonoma de Madrid

e-mail: mugarte@cbm.uam.es

- **Alberto Burlina**

Pediatrics Dept. Azienda Ospedale Universita of Padua

e-mail: burlina@pediatria.unipd.it

- **Laura F. T. Vilarinho**

National Neonatal Screening Laboratory, INSA Research Unit

e-mail: laura.vilarinho@insa.min-saude.pt

Experimental evidence that the major metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency induce hypophosphorylation of cytoskeletal proteins of neural cells mediated by protein kinase inhibition and oxidative stress

Carolina Gonçalves Fernandes[a], Paula Pierozan[a], Gilberto Machado Soares[a], Fernanda Ferreira[a], Ângela Zanatta[a], Alexandre Umpierrez Amaral[a], Clarissa Günther Borges[a], Moacir Wajner[a,b] and Regina Pessoa-Pureur[a*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Porto Alegre, RS, Brazil

[b] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

* Corresponding Author: Dr. Regina Pessoa-Pureur

Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Departamento de Bioquímica, Rua Ramiro Barcelos 2600 anexo, CEP 90035-003 Porto Alegre - RS, BRASIL, Fax: 5551 3308 5535, Tel: 5551 3308 5565; E-mail: rpureur@ufrgs.br

Abstract

3-Hydroxy-3-methylglutaryl-CoA lyase (HL) deficiency is an inborn disease biochemically characterized by tissue accumulation and high urinary excretion of 3-hydroxy-3-methylglutaric (HMG), 3-methylglutaric (MGA), 3-methylglutaconic (MGT) acids and also of 3-hydroxyisovaleric acid to a lesser extend. Neurological symptoms and abnormalities are commonly observed in patients with this disease, but their pathogenesis is poorly known. In the present study we found that HMG, MGA and MGT caused hypophosphorylation of glial fibrillary acidic protein and the neurofilament subunits NFL, NFM and NFH in striatal and cortical slices of rats. Energy deprivation was not responsible for these effects since important parameters of mitochondrial energy production were not altered. However, HMG-induced hypophosphorylation of cytoskeletal proteins was mediated by inhibition of protein kinases, with no alterations of protein phosphatases. Thus, we demonstrated that protein cAMP-dependent kinase (PKA) inhibition was involved in the hypophosphorylation of Ser55 of NFL, as well as JNK/MAPK inhibition resulted in the hypophosphorylation of KSP repeats of NFM and NFH subunits. It was also observed that the cytoskeletal hypophosphorylation was dependent on synaptic and extra synaptic (NR2B subunit) NMDA receptors and also on Ca^{2+}. Furthermore, the NOS inhibitor, L-NAME, and the antioxidant, TROLOX (polar analog of vitamin E), fully prevented the hypophosphorylation and the inhibition of PKA and JNK activities caused by HMG. These findings indicate that the principal metabolites accumulating in HL deficiency disrupt the cytoskeleton phosphorylating system probably through NMDA receptors, Ca^{2+} and disruption of redox homeostasis. It is presumed that these mechanisms may contribute to the neuropathology of this disease.

Keywords: 3-hydroxy-3-methylglutaryl-CoA lyase deficiency; hyporphosphorylation; intermediate filaments; cell signaling; glutamatergic receptors

Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) lyase (EC 4.1.3.4) is an enzyme found in the mitochondrial matrix and peroxisomes that catalyzes the final step in the metabolism of L-leucine also participating in ketone body synthesis (Gibson et al., 1988; Wysocki and Hähnel, 1986). Deficiency of HMG-CoA lyase (HL), also called 3-hydroxy-3-methylglutaric aciduria (HMGA, OMIM 246450), is an autosomal recessive inborn disease with an estimated prevalence of 1 in 100,000 live births (Pié et al., 2007; Reimão et al., 2009). This enzymatic deficiency results in tissue accumulation and high urinary excretion of 3-hydroxy-3-methylglutaric (HMG), 3-methylglutaric (MGA), 3-methylglutaconic (MGT) and 3-hydroxyisovaleric acids (Faull et al., 1976). The symptoms appearance occurs in the first year of life, normally after a fasting period or other catabolic event. The most prominent findings are vomiting, hepatomegaly with mild liver alterations, lethargy, coma, convulsions, metabolic acidosis and hypoketotic hypoglycaemia (Gibson et al., 1988). Encephalopathy with generalized cerebral edema, structural abnormalities in the white matter (hypomyelinization) of cerebral cortex and the basal ganglia are the main findings on MRI (Yalcinkaya et al., 1999; Yylmaz et al., 2006; Zaifeiriou et al., 2007).

Recent *in vivo* studies described oxidative damage and activation of N-methyl-D-aspartate (NMDA) receptors with free radical formation caused by HMG and MGA in the CNS of rats (Fernandes et al., 2013; da Rosa et al., 2013), supporting a role for excitotoxicity and redox imbalance in the brain damage in this disorder. In this context, it must be stressed that these metabolites are structurally similar to glutamate. It is also of note that NMDA receptors and oxidative imbalance are implicated in the misregulation of signaling pathways disrupting the phosphorylating system associated

with the cytoskeleton in various pathological conditions (Loureiro et al., 2010a,b; Cattani et al., 2013; Pierozan et al., 2010).

Intermediate filament (IF) proteins are cell-specific constituents of the cytoskeleton. Mature astrocytes express glial fibrillary acidic protein (GFAP), which is necessary to maintain cell shape, motility/migration, proliferation, glutamate homeostasis and neurite outgrowth, being also important for protection against CNS injury (Middeldorp and Hol, 2011). Neurons express neurofilaments (NF), which are constituted by three distinct subunits classified by their molecular masses: NFH (high molecular weight neurofilament subunit), NFM (middle molecular weight neurofilament subunit) and NFL (light molecular weight neurofilament subunit). They represent the most abundant cytoskeletal elements in mature neurons where their principal function is to control the axonal caliber and nerve conductivity (Perrot and Eyer, 2009).

The physiological role of IF proteins is mainly controlled by phosphorylation, which is a dynamic process mediated by the combined action of several protein kinases and phosphatases in response to extracellular signals. In this context, the phosphorylation sites in IF subunits are located in their amino- and carboxyl-terminal domains and the phosphorylation levels are implicated in the association/disassociation ability as well as in the interaction with other cytoskeletal components (Sihag et al., 2007). *In vivo* and *ex vivo* studies from our group and others demonstrated that the phosphate groups on the N-terminal head domain of GFAP, vimentin, and NFL are added by second messenger-dependent protein kinases, such as cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (PKCaMII), and protein kinase C (PKC) (Sihag et al., 2007; Pierozan et al., 2010, 2012). Phosphorylation sites in the C-terminal domain of NFM and NFH are located in Lys-

Ser-Pro (KSP) repeat regions of these subunits. The KSP repeats are phosphorylated by proline directed kinases such as Cdk5 (Guidato et al., 1996), the mitogen-activated protein kinases (MAPK) Erk1/2, JNK, and p38MAPK (Veeranna et al., 1998; Brownlees et al., 2000) and by the glycogen synthase kinase 3 (GSK3) (Veeranna et al., 2000).

Protein phosphatases are highly concentrated in the mammalian brain (Strack et al., 1997) and the cytoskeleton is a preferential target for the action of Ser-Thr phosphatases 1, 2A and 2B (PP1, PP2A and PP2B) (de Almeida et al., 2003; Funchal et al., 2005a,b; de Mattos-Dutra et al., 1997). In this context, PP1 and PP2B-mediated hypophosphorylation of IF proteins in the cerebral cortex of rats caused by intrastriatally injected of quinolinic acid (QUIN) was first reported in our laboratory and proposed to misregulate cytoskeletal homeostasis and be associated with QUIN toxicity (Pierozan et al., 2014).

Thus, considering that the phosphorylating system associated with the cytoskeleton may be related to neural damage and that the pathogenesis of the brain damage in HL deficiency is still poorly elucidated, in the present report we investigated the actions of HMG, MGA and MGT on the phosphorylating system associated with the IF-enriched cytoskeleton in striatal and cortical slices of young rats. We also searched for the role of NMDA receptors and oxidative stress on the alterations of the cytoskeletal phosphorylating system homeostasis provoked by HMG exposure. We hypothesize that disruption of cytoskeletal homeostasis could be implicated in the neural damage induced by the main metabolites accumulating in HMG-CoA lyase deficiency.

Material and Methods

Chemicals

[^{32}P]Na_2HPO_4 was purchased from Comissão de Energia Nuclear (CNEN), São Paulo, Brazil. 3-Hydroxy-3-methylglutaric acid (HMG), 3-methylglutaconic (MGA) acid, 3-methylglutaconic acid (MGT), acrylamide, antipain, 1,2-bis (2-aminophenoxy) ethane-N-N-N'-N'-tetraacetic acid tetrakis (acetoxy-methyl Ester) (BAPTA-AM), benzamidine, bisacrilamide, calyculin A, chymostatin, dizocilpine (MK-801), ethylene glycol tetraacetic acid (EGTA), ifenprodil, leupeptin, NG-nitro-L-arginine methyl ester (L-NAME), N-acetyl-L-cystein (NAC), pepstatin, tacrolimus (FK-506), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX) were obtained from Sigma (St. Louis, MO, USA). Anti-pNFL(Ser55) and anti-p38MAPK and anti-phospho p38MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-PKCamII α, anti-JNK (p54), anti-JNK (p46) anti-pJNK (p54), anti-pJNK (p46), anti-Erk (p44), anti-Erk (p42) anti-pErk (p44), anti-pErk (p42) and anti PKAcα antibodies were from Cell Signaling (Danvers, MA, USA). The antibody NFH (anti-KSP repeats) was purchased from Chemicon (Temecula, CA, USA). The chemiluminescence ECL kit peroxidase and the conjugated anti-rabbit IgG were obtained from Amersham (Oakville, Ontario, Canada). All chemicals were of analytical grade.

Animals

Thirty-day-old Wistar rats obtained from Central Animal House of the Departmento de Bioquímica da Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, were used in the experiments. The animals were maintained on 12:12 h light/dark cycle in an air-conditioned constant temperature (22 °C ± 1 °C) colony room, with food and water

ad libitum. The project followed the experimental protocol "Principles of Laboratory Animal Care" (NHI publication 85-23, revised 1985) and was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul.

Phosphorylation assay

Pre-incubation

Rats were sacrificed by decapitation, the striatum and cerebral cortex were dissected onto Petri dishes placed on ice and cut into 400 μm thick slices with a McIlwain chopper. Tissue slices were initially preincubated at 30 °C for 10 min in a Krebs-Hepes medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-Hepes, 12 mM glucose, 1 mM $CaCl_2$, pH 7.4, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM pepstatin and 0.7 μM chymostatin. In some experiments we used 50 μM BAPTA-AM, 0.2 μM caliculyn, 1 mM EGTA, 100 μM FK-506, 10 μM ifenprodil, 1 mM Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), 10 μM dizocilpine (MK-801), 1 mM N-acetyl cysteine (NAC), 5 μM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX).

Incubation and extraction of the cytoskeletal fraction

After pre-incubation, the medium was changed by 100 μL of a Krebs-Hepes medium in the absence or presence of HMG, MGA or MGT (0.2 to 5 mM). In some experiments we evaluated the effects of 5 mM HMG using striatum slices in order to find out the mechanisms of action of this organic acid on the cytoskeletal phosphorylating system. 100 μCi of [^{32}P]-orthophosphate was then added to the incubation medium and the labeling reaction was allowed to proceed for 60 min at 30 °C. Reaction was stopped with 1 mM of cold stop buffer (150 mM NaF, 5 mM EDTA, 5 mM EGTA, 50 mM Tris-

HCl, pH6.5, and the protease inhibitors described above). Slices were then washed twice with stop buffer to remove excess radioactivity. These slices were homogenized in 400 μL of ice-cold high salt buffer containing 5 mM KH_2PO_4, 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1 % triton X-100, pH 7.1 and the protease inhibitors described above. The homogenate was centrifuged at 15,800 x g for 10 min at 4 °C, the supernatant discarded and the pellet washed/resuspended with the same volume of the high salt buffer. The washed/resuspended pellet was centrifuged as described and the supernatant was discarded. The Triton-insoluble IF-enriched pellet, containing NF subunits and GFAP, was dissolved in 1 % SDS and after prepared to electrophoresis with a concentrated sample buffer containing 33.3 % glycerol, 16 % β-mercaptoethanol, 5 % SDS, 0.033 M NaOH and boiled for 3 min (Funchal et al., 2003). Samples were analyzed in 7.5 % SDS-PAGE

Polyacrylamide gel electrophoresis (SDS-PAGE)

Samples of IF-enriched fraction containing equal protein content (50 μg) were loaded onto a 7.5 or 10 % gel. The gels were exposed to X-ray films (T-mat G/RA) (Kodak) at -70 °C with intensifying screens and after 30 days the autoradiograph was obtained. The [^{32}P]-orthophosphate incorporated to each sample was quantified from each correspondent cytoskeletal protein by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an AlphaEaseFC version 6.0.0 software (FluorChem SA). Protein loading was controlled by Comassie blue R staining. All bands measured were within the linear range of the film.

Western blot analysis

Total tissue homogenate was obtained from striatum slices previously incubated for 60 min at 30 °C with or without 5 mM HMG and in some experiments with the addition of

1 mM of L-NAME or 5 μM of TROLOX. The slices were homogenized in 100 mL of a lysis solution containing 2 mM EDTA, 50 mM Tris-HCl, 4 % SDS, pH 6.8. This was followed by dilution in a solution containing 40 % glycerol, 5 % β-mercaptoethanol, 50 mM Tris-HCl, pH 6.8 and boiled for 3 min. The samples (30 μg of protein) were loaded onto a 10 % gel by SDS-PAGE according to the discontinuous system of Laemmli (Laemmli, 1970) and transferred to nitrocellulose membranes (Trans-blot SD semi-dry transfer cell, BioRad) for 1 h at 15 V in transfer buffer: 48 mM Trizma, 39 mM glycine, 20 % methanol and 0.25 % SDS. The nitrocellulose membranes were washed for 10 min in Tris buffered saline (TBS: 0.5 M NaCl, 20 mM trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5 % deffated dried milk). After incubation, the blots were washed twice for 5 min with TBS plus 0.05 % Tween-20 (T-TBS), and then incubated overnight at 4 °C in blocking solution containing the following monoclonal antibodies: anti-PKCaMII α, anti-p38/MAPK, anti-PKAcα, anti-JNK (p54), anti-JNK (p46) anti-pJNK (p54), anti-pJNK (p46), anti-Erk (p44), anti-Erk (p42) anti-pErk (p44), anti-pErk (p42) diluted 1:1000, anti-pNFL(Ser55) diluted 1:800, anti-NFH/NFM KSP repeats diluted 1:100 anti-β actin diluted 1:1000. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in TBS containing peroxidase conjugated rabbit anti-mouse IgG or anti-rabbit IgG diluted 1:2000. We also used the following negative controls: antibody negative control (lacking the sample containing the antigen of interest) and sample negative control (lacking the first antibody). The blot was washed twice again for 5 min with T-TBS and twice for 5 in with TBS. The blot was then developed using a chemiluminescence ECL kit and quantified as described above.

Cell viability

Cell viability was evaluated by lactate dehydrogenase (LDH) activity (Whitaker and McKay, 1969) and by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction (Liu et al., 1997) in striatal and cortical slices. LDH release was determined using a colorimetric commercial kit (from Doles, Brazil) and expressed as percentage of positive control samples after addition of 10 % Triton X-100. Results were expressed relative to 100% LDH activity. For the MTT assay, MTT (0.5 mg/mL) in Krebs–Hepes medium was incubated for 60 min at 30 °C. The tetrazolium ring of MTT is cleaved by various dehydrogenases and then precipitated as a blue formazan product. The medium was then aspirated, the precipitated formazan was solubilized with dimethyl sulfoxide and quantified spectrophotometrically at a wavelength of 550 nm.

Parameters of energy homeostasis

CO_2 production

Cerebral cortex and striatum slices (400 μm) were pre-incubated with or without 5 mM HMG or MGA in Krebs–Ringer bicarbonate buffer (1.17 mM KH_2PO_4, 118 mM NaCl, 4.69 mM KCl, 24.1 mM $MgSO_4$, 3.33 mM $CaCl_2$ and 650 mM $NaHCO_3$, pH 7.4) into small flasks (11 cm^3) in a volume of 450 μL at 35 °C for 60 min in a metabolic shaker (90 oscillations / min). After pre-incubation, [U-^{14}C] glucose (0.055 mCi) plus 5.0 mM of unlabeled glucose was added to the incubation medium. The flasks were gassed with an O_2/CO_2 (95:5) mixture and sealed with rubber stoppers Parafilm M. Glass center wells containing a folded 60 nm / 4 nm piece of Whatman 3 filter paper were hung from the stoppers. After incubation in the metabolic shaker 0.2 mL of 50 % trichloroacetic acid was supplemented to the medium and 0.1 mL of benzethonium hydroxide was

added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete CO_2 trapping and then opened. The filter paper were removed and added to vials containing scintillation fluid, and radioactivity was counted (Reis de Assis et al., 2004). Results were calculated as nmol CO_2 . h^{-1} . g tissue $^{-1}$ and expressed as percentage of control.

Preparation of mitochondrial fractions

Mitochondrial preparations from forebrain were isolated according to Rosenthal and co-workers (1987), with slight modifications (Mirandola et al., 2008). Animals were decapitated, the forebrain was dissected and homogenized with a glass hand-held homogenizer in ice-cold mitochondria isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1 % bovine serum albumin (BSA, free fatty acid) and 10 mM HEPES, pH 7.2. The homogenate was centrifuged at 2,000 x g for 3 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 12,000 x g for 8 min at 4 °C. The resultant pellet was resuspended in 5 mL of isolation buffer containing 20 μL of 10% digitonin (final concentration of 0.04%), and centrifuged 12,000 x g for 10 min at 4 °C. The supernatant was discarded and the pellet resuspended in 5 mL of isolation buffer without EGTA and centrifuged at 12,000 x g for 10 min at 4 °C. The final pellet was resuspended in isolation buffer without EGTA in an approximate protein concentration of 15-20 mg/mL. Protein concentration was measured by the method of Bradford (1976) using BSA as standard. This preparation results in a mixture of synaptosomal and non-synaptosomal mitochondria similar to the general brain composition. Mitochondria obtained from forebrain were used in the assays immediately after isolation and assays were carried out in the absence or presence of Ca^{2+}.

Spectrophotometric analysis of the citric acid cycle enzyme activities

The activities of aconitase and α-ketoglutarate dehydrogenase (α-KGDH) complex enzymes of the citric acid cycle (CAC) were determined using enriched mitochondrial fractions from whole brain, whereas succinate dehydrogenase (SDH) was measured in brain homogenates. HMG or MGA (5 mM) were supplemented to the subcellular preparations and submitted to a pre-incubation at 37 °C for 60 min. The activity of the enzyme aconitase (ACO) was measured according to Morrison (1954), following the reduction of $NADP^+$ at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of the α-KGDH complex was evaluated according to Lai and Cooper (1986) and Tretter and Adam-Vizi (2004), with slight modifications (Viegas et al., 2009). The reduction of NAD^+ was recorded in a Hitachi F-4500 spectrofluorometer at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of succinate dehydrogenase (SDH) was determined as described by Fischer and colleagues (1985). The activities of these citric acid cycle enzymes were calculated as nmol . min^{-1} . mg $protein^{-1}$; mmol . min^{-1} . mg $protein^{-1}$ or mmol . min^{-1} . mg $protein^{-1}$ and expressed as percentage of control.

Spectrophotometric analysis of the respiratory chain complexes I–IV activities

The activities of succinate–2,6-dichloroindophenol (DCIP)–oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complexes II–III) were determined in homogenates from striatum or cerebral cortex according to Fischer and co-workers (1985) and that of cytochrome *c* oxidase (complex IV) according to Rustin and co-workers (1994). The methods described to measure these activities were slightly modified, as described in details in a previous report (da Silva et al., 2002). HMG or MGA (5 mM) was added to the reaction medium at the beginning of the assays, while

no metabolite was added to controls. The activities of the respiratory chain complexes were calculated as nmol . min^{-1} . mg $protein^{-1}$ or mmol . min^{-1} . mg $protein^{-1}$ and expressed as percentage of control.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi m$) was estimated according to Akerman and Wikstrom (1976) and Kowaltowski and co-workers (2002). The fluorescence of 5 μM cationic dye safranine O, a $\Delta\Psi m$ indicator, was followed at excitation and emission wavelengths of 495 and 586 nm. CCCP was added in the end of measurements to abolish $\Delta\Psi m$. Mitochondrial incubations were carried out at 37 °C, with continuous magnetic stirring. The assay was conducted in medium containing 150 mM KCl, 5 mM $MgCl_2$, 30 μM EGTA, 0.1 mg / mL BSA, 5 mM HEPES, 2 mM KH_2PO_4, pH 7.2, 1 μg / mL oligomycin A, using state 4 respiring mitochondria (0.5 mg protein / mL) supported by 2.5 mM glutamate plus 2.5 mM malate or succinate plus rotenone. HMG (5mM), MGA (5mM), $CaCl_2$ (30 μM) and CCCP (3 μM) were added as indicated by the arrows in the figure. Traces are representative of independent experiments carried out in mitochondrial preparations from forebrain of three animals and were expressed as fluorescence arbitrary units (FAU).

Protein determination

The protein concentration was determined by the methods of Lowry (Lowry et al., 1951) or Bradford (Bradford, 1976) using serum albumin as standard.

Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F-test was significant. A $p < 0.05$ was

considered significant. All analyses were performed using the SPSS 19.0 software program on an IBM-PC compatible computer.

Results

We first observed that cell viability (MTT assay and LDH release) was not altered in striatal and cortical slices exposed to HMG, MGA or MGT for 60 min (results not shown). In contrast, HMG (Figure 1 A, B, G and H) and MGA (Figure 1 C, D, I and J) caused hypophosphorylation of GFAP and NF subunits (NFL, NFM and NFH) in both brain structures, whereas MGT provoked hypophosphorylation of cytoskeletal IF only in the striatum (Figure 1E and K).

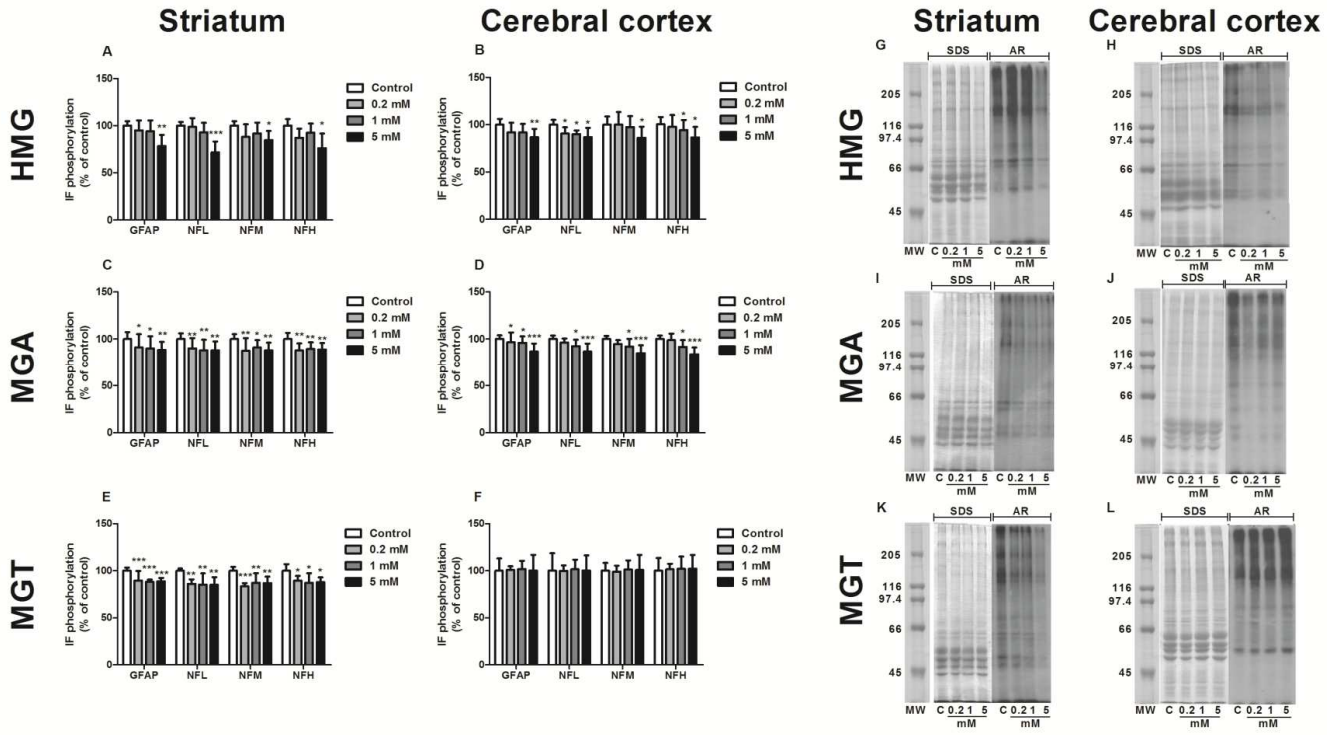


Figure 1 –Effect of HMG, MGA or MGT on the phosphorylation levels of IFs in the cytoskeletal fraction from slices of striatum or cerebral cortex of 30-day-old rats. Tissue slices were incubated during 60 min in the presence or absence (control group) of the metabolites (0.2 – 5 mM). Effects of HMG (A and B), MGA (C and D) and MGT (E and F) on the radioactivity incorporated into IFs in striatal and cortical slices. Data are reported as mean ± SD of 18 animals in each group and expressed as percentage of controls. Statistically significant from controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Duncan's multiple range test). Comassie blue stained molecular weight standards, representative stained gel and corresponding autoradiography from striatal slices on HMG (G), MGA (I) and MGT effects (K) and from cerebral cortex slices on HMG (H), on MGA (J) and MGT effects (L). AR: autoradiography; C: Control; MW: molecular weight standard proteins; SDS: SDS page.

In order to understand the mechanisms underlying the effects of HMG and MGA on the phosphorylation system associated with the cytoskeleton, we measured some parameters of energy metabolism to test the possibility that decreased ATP availability could be responsible for the disrupted phosphorylating system. We found no alteration of citric acid cycle enzyme activities, CO_2 production, activities of the respiratory chain complexes II, II-III and IV and mitochondrial membrane potential in striatal and cortical preparations exposed to HMG- and MGA, making unlikely that decreased energy

production could explain our results of hypophosphorylation (Tables I, II and III; Figure 2).

Table I – Effect of 3-hydroxy-3-methylglutaric (HMG) or 3-methylglutaric (MGA) acids on the CO_2 production from [U-^{14}C] glucose in rat brain tissue slices

	Controle	HMG	MGA
Striatum	100 ± 13.1	113 ± 6.15	-
Cerebral cortex	100 ± 26.9	114 ± 40.4	122 ± 39.1

Values are mean ± standard deviation for four to six independent (animals) experiments per group. The CO_2 production was calculated as pmol $^{14}CO_2$. h^{-1} . g $tissue^{-1}$ and expressed as percentage of control. No significant differences between groups were detected (one way ANOVA).

Table II – Effect of 3-hydroxy-3-methylglutaric (HMG) or 3-methylglutaric (MGA) acids on the citric acid cycle (CAC) enzymes in enriched mitochondrial fractions from rat brain

	Control	HMG	MGA
ACO	100 ± 26.3	113 ± 20.9	110 ± 17.7
α-KGDH	100 ± 42.2	98.1 ± 34.6	103 ± 54.8

Values are mean ± standard deviation for four to six independent (animals) experiments per group. The activities of aconitase (ACO) and α-ketoglutarate dehydrogenase (α-KGDH) were calculated as μmol NADPH . min^{-1} . mg $protein^{-1}$ and mmol NADH . min^{-1} . mg $protein^{-1}$, respectively, and expressed as percentage of control. No significant differences between groups were detected (one way ANOVA).

Table III - Effect of 3-hydroxy-3-methylglutaric (HMG) or 3-methylglutaric (MGA) acids on the activities of respiratory chain complexes and the activity of the CAC enzyme succinate dehydrogenase (SDH) in rat brain homogenates

	Striatum			Cerebral cortex		
	Control	HMG	MGA	Control	HMG	MGA
II	100 ± 14.2	101 ± 13.4	97.8 ± 6.29	100 ± 5.50	95.9 ± 12.7	97.8 ± 7.25
II-III	100 ± 11.8	100 ± 8.46	101 ± 9.13	100 ± 7.33	99.3 ± 3.07	101 ± 6.61
IV	100 ± 10.5	100 ± 17.4	94.4 ± 11.5	100 ± 29.1	99.9 ± 10.4	102 ± 15.2
SDH	100 ± 7.46	100 ± 18.3	98.7 ± 11.3	100 ± 11.3	96.1 ± 4.83	98.3 ± 2.89

Values are mean± standard deviation for four to six independent (animals) experiments per group. The activities of complexes II, II–III, IV and the activity of succinate dehydrogenase (SDH) were calculated as nmol . min^{-1} . mg $protein^{-1}$ and nmol DCIP . min^{-1} . mg $protein^{-1}$, respectively, and expressed as percentage of control. No significant differences between groups were detected (one way ANOVA).

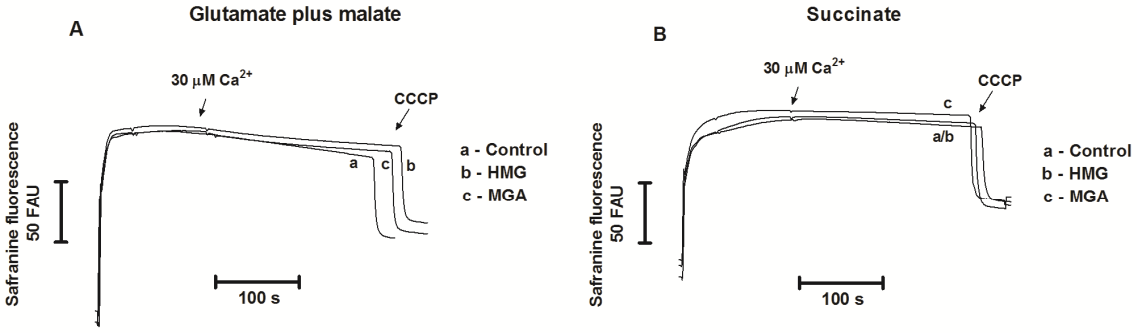


Figure 2 – Effects of HMG or MGA acids on mitochondrial membrane potential in the presence of Ca^{2+} (30 μM). All experiments were performed in a reaction media containing mitochondrial preparations (0.5 mg protein. mL^{-1}) supported by glutamate plus malate (A) or succinate (B). HMG or MGA (5 mM, lines b-c, respectively) were added 50 seconds after the beginning of the assay. Controls (line a) were performed in the absence of the metabolites. The reaction media contained 30 μM EGTA. CCCP (3 μM) was added at the end of the assays. Traces are representative of 3 independent experiments (animals) and were expressed as fluorescence arbitrary units (FAU).

We further investigated the mechanisms underlying the hypophosphorylation of IF induced by HMG, the major accumulating compound, using the striatum that is mainly compromised in patients affected by HL deficiency (Sweetman and Williams, 2001; van der Knaap et al., 1998; Yalcinkaya et al., 1999; Yylmaz et al., 2006).

Since hypophosphorylation can be due to protein phosphatase activation and/or protein kinase inhibition, we first searched for the protein phosphatases involved in the hypophosphorylation of cytoskeletal proteins using specific Ser-Thr phosphatase inhibitors. We observed that the potent PP1 and PP2A inhibitor calyculin A (Sheppek et al., 1997) and the specific PP2B inhibitor FK506 (Baba et al., 2003) were ineffective in preventing HMG-induced decrease of IF phosphorylation (Figure 3).

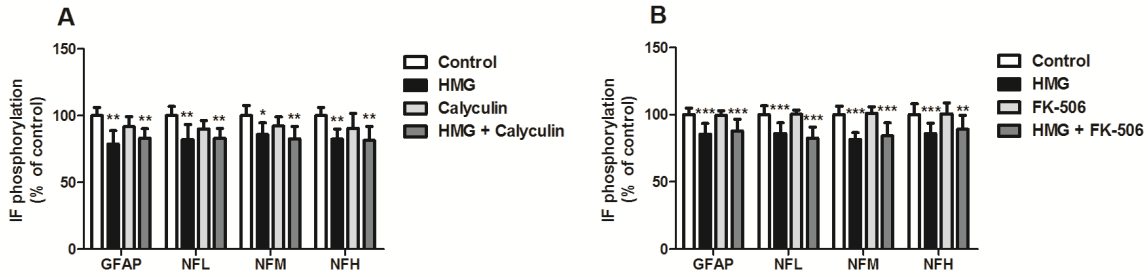


Figure 3 – Effects of PP1, PP2A and PP2B inhibitors on HMG-induced decrease of the phosphorylation levels of IFs in the cytoskeletal fraction from striatum slices of 30-day-old rats. Tissue slices were co-incubated during 60 min in the presence or absence (control group) of the metabolite (5 mM) plus 0.2 μM calyculin (A; PP1and PP2A inhibitor) or 100 μM FK-506 (B; PP2B inhibitor). Data are reported as mean ± SD of 18 animals in each group and expressed as percentage of controls. Statistically significant differences from controls were determined by one-way ANOVA followed by Duncan's multiple range test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control.

We next investigated whether the most common Ser–Thr kinases known to modulate the phosphorylating level of IF cytoskeletal proteins, namely cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase (PKCamII) and the mitogen-activated protein kinases (MAPK) Erk, JNK and p38MAPK (Hisanaga et al., 1990; Sihag and Nixon, 1989; Sihag et al., 2007), were involved in HMG hypophosphorylating effect. Western blot analysis using specific antibodies showed unaltered immunocontent of the active PKCamII subunit (Figure 4A), but decreased immunocontent of the active PKA subunit (Figure 4B), evidencing a role for PKA in the hypophosphorylation of the IF proteins induced by this metabolite. We also tested the phosphorylation level of NFLSer55, known to be the preferential phosphorylation site for PKA in the amino-terminal region of NFL subunit (Grant and Pant, 2000). It was observed reduced immunocontent of phosphorylated Ser55, reinforcing the involvement of PKA in HMG hypophosphorylating effects (Figure 4C).

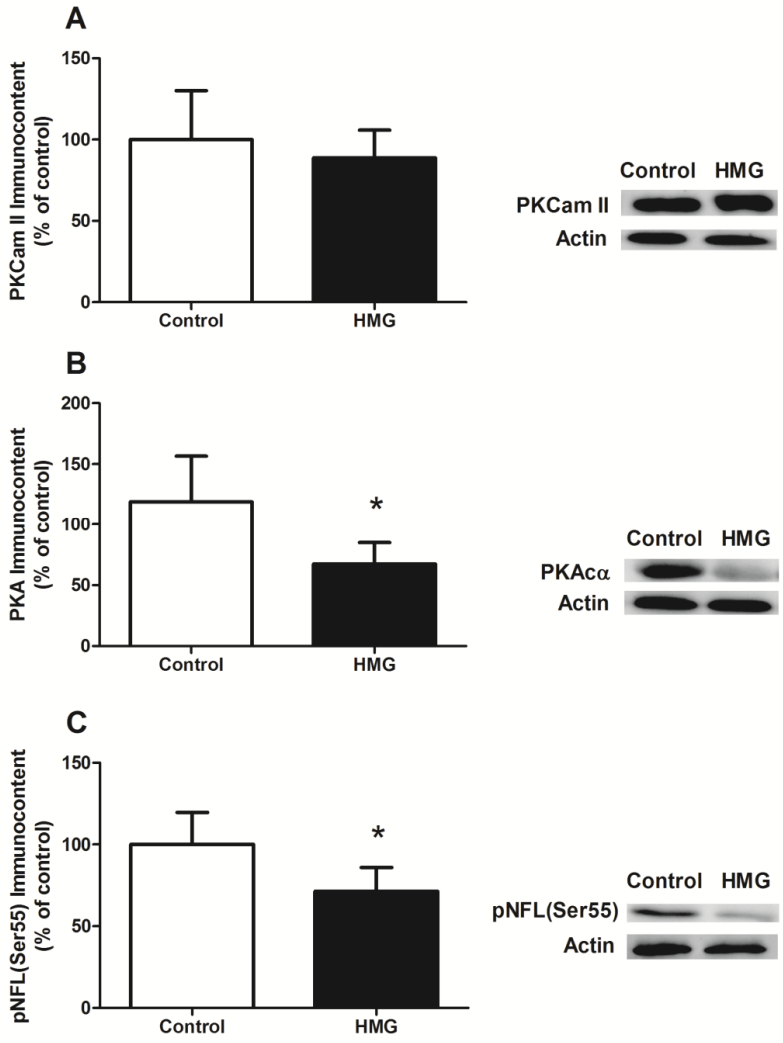


Figure 4 - Effects of HMG on the immunocontent of PKCamII (A), PKAC α (B) and Ser55 phosphorylation site (C) in the total homogenates from striatal slices of 30-day-old rats. Western blot with anti-PKCamII α, anti-PKAcα or anti-pNFL(Ser55) antibodies. Tissue slices were incubated during 60 min in the presence or absence (control group) of the metabolite (5 mM). Data are reported as mean ± SD of 6 animals in each group and expressed as percentage of controls. Statistically significant differences from controls were determined by Student *t* test. * $p < 0.05$ compared with control.

Next, we examined the involvement of the MAPK cascade on HMG-induced IF hypophosphorylation. Western blot analysis with specific monoclonal antibodies showed unaltered immunocontent of Erk1/phospho-Erk1 and Erk2/phospho-Erk2 (Figure 5A), as well as of p38MAPK/phospho-p38MAPK (Figure 5B). In contrast,

phospho-JNK, but not total JNK, immunocontent was significantly decreased (Figure 5C), implying a reduction of JNK activity/phosphorylation. In an attempt to verify the implications of the downregulated pJNK on the hypophosphorylation of NFM and NFH subunits, we determined the phosphorylation level of the KSP repeats located in the tail domain of these subunits, known to be the preferential phosphorylating sites for MAPKs (Veeranna et al., 2000). Results of Western blot assay with the phosphoKSP repeat antibody showed reduced immunocontent of these phosphorylated sites in response to HMG (Figure 5D).

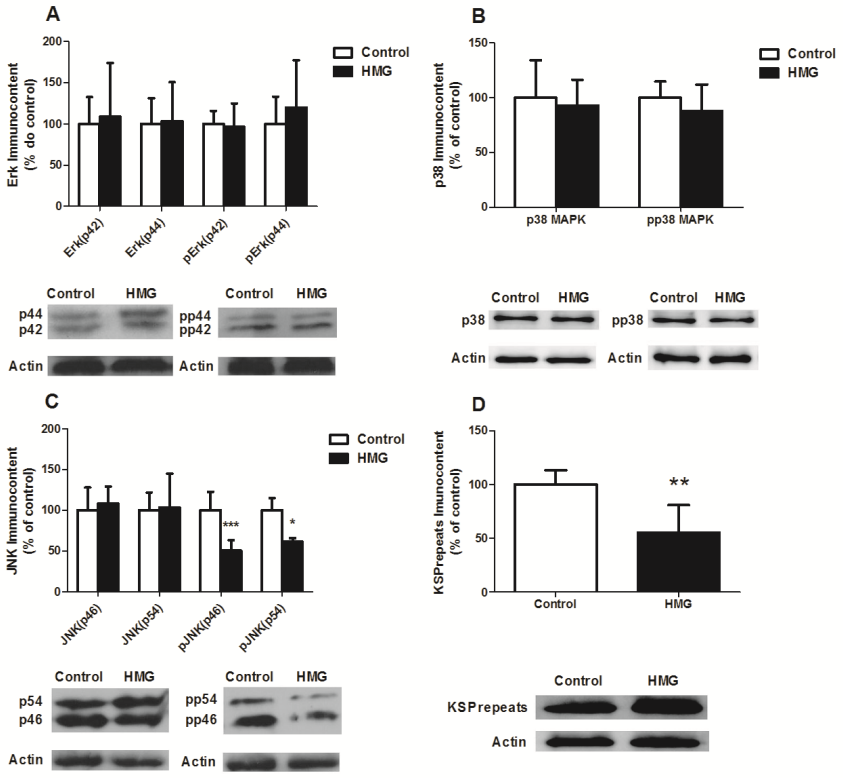


Figure 5 - Effects of HMG on the immunocontent of Erk 1/2 (A), p38MAPK (B), JNK (C) and phosphoKSP repeats (D) in the total homogenates from striatal slices of 30-day-old rats. Western blot with anti-Erk (p42), anti-Erk (p44), anti-pErk (p42), anti pErk (p44), anti-p38/MAPK, anti-phospho-p38/MAPK, anti-JNK (p54), anti-JNK (p46), anti-pJNK (p54), anti-pJNK (p46) or anti-KSP repeat antibodies. Tissue slices were incubated during 60 min in the presence or absence (control group) of the metabolite (5 mM). Data are reported as mean ± SD of 6 animals in each group and expressed as percentage of controls. Statistically different from controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t* test).

Since we have shown that NMDA receptors are implicated in oxidative stress caused by intracerebral administration of HMG in rat striatum (Fernandes et al., 2013), we next assessed the involvement of NMDA receptors in the IF hypophosphorylated levels in HMG treated striatal slices. Results showed that 10 μM MK-801, a non-competitive antagonist of synaptic NMDARs (Pierozan et al., 2010) totally prevented HMG-induced GFAP, NFL, NFM and NFH hypophosphorylation (Figure 6A). Similar results were obtained with 10 μM ifenprodil, a selective antagonist of extra-synaptic

NMDAR-containing NR2B subunit (Rittase et al., 2014) (Figure 6B). These data indicate the participation of both synaptic and extra-synaptic glutamate receptors in the hypophosphorylating effect of HMG towards IF.

We also found that the IF hypophosphorylation caused by HMG was totally prevented when striatum slices were co-incubated with the intracellular and extracellular Ca^{2+} chelators BAPTA-AM (50 μM) and EGTA (1 mM), respectively, in a Ca^{2+}-free medium (Figure 6C). These findings support a role for Ca^{2+} underlying the actions of HMG in the homeostasis of the cytoskeleton associated phosphorylating system.

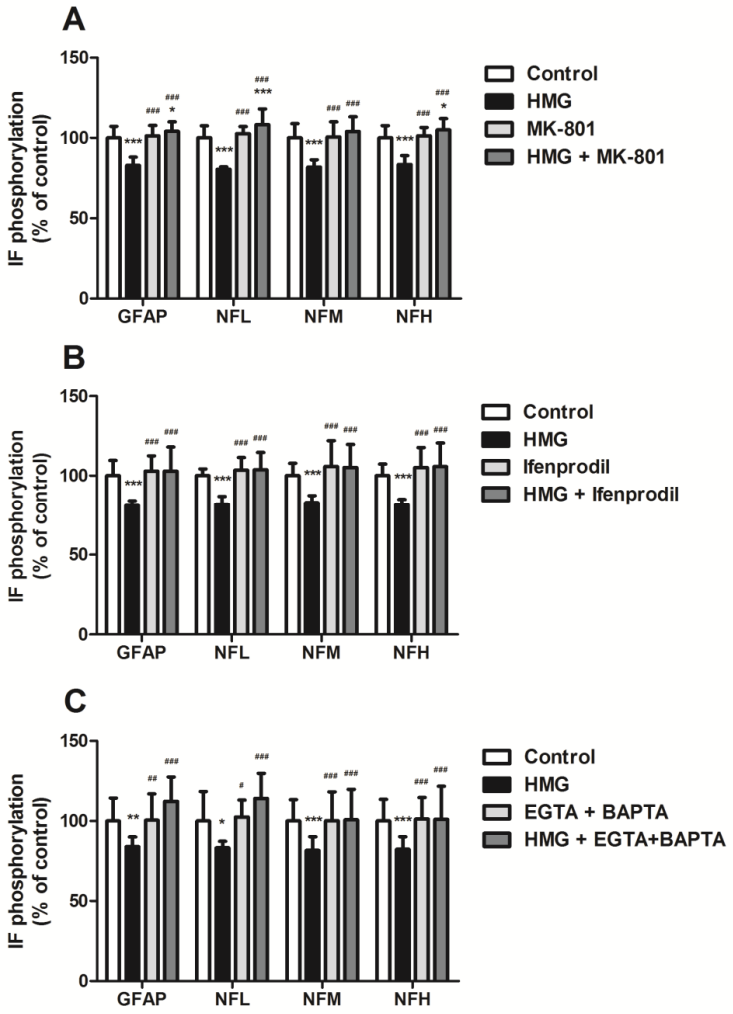


Figure 6 – Effects of a synaptic and extra-synaptic NMDARs and Ca^{2+} levels on the effect of HMG on the phosphorylation level of IF proteins from striatal slices of 30-day-old rats. Tissue slices were co-incubated during 60 min in the presence or absence (control group) of the metabolite (5 mM) plus the synaptic NMDAR antagonist MK-801 (A; 10 μM), the extra-synaptic NMDAR inhibitor (NR2B subunit) ifenprodil (B; 10 μM) or the intra and extra-cellular Ca^{2+} chelators BAPTA-AM, (50 μM) and EGTA, (C;1 mM), respectively. Data are reported as mean ± SD of 18 animals in each group and expressed as percentage of controls. Statistically significant differences from controls were determined by one-way ANOVA followed by Duncan's multiple range test. * $p < 0.05$, *** $p < 0.001$ compared with control; $^{###}$ $p < 0.001$ compared with HMG.

Next, we evaluated the role of reactive species in the hypophosphorylating effects of HMG. We observed that the nitric oxide synthase (NOS) inhibitor L-NAME (1 mM) totally prevented the hypophosphorylation of GFAP and NF subunits (Figure

7A), suggesting a role for reactive nitrogen species in such effect. TROLOX (5 μM), a soluble polar vitamin E analog, also normalized the phosphorylating level of GFAP and NF subunits (Figure 7B), suggesting a role for peroxyl radicals in the HMG action. In contrast, N-acetyl cysteine (NAC) (1 mM), a GSH precursor, failed to prevent HMG-elicited IF hypophosphorylation (Figure 7C).

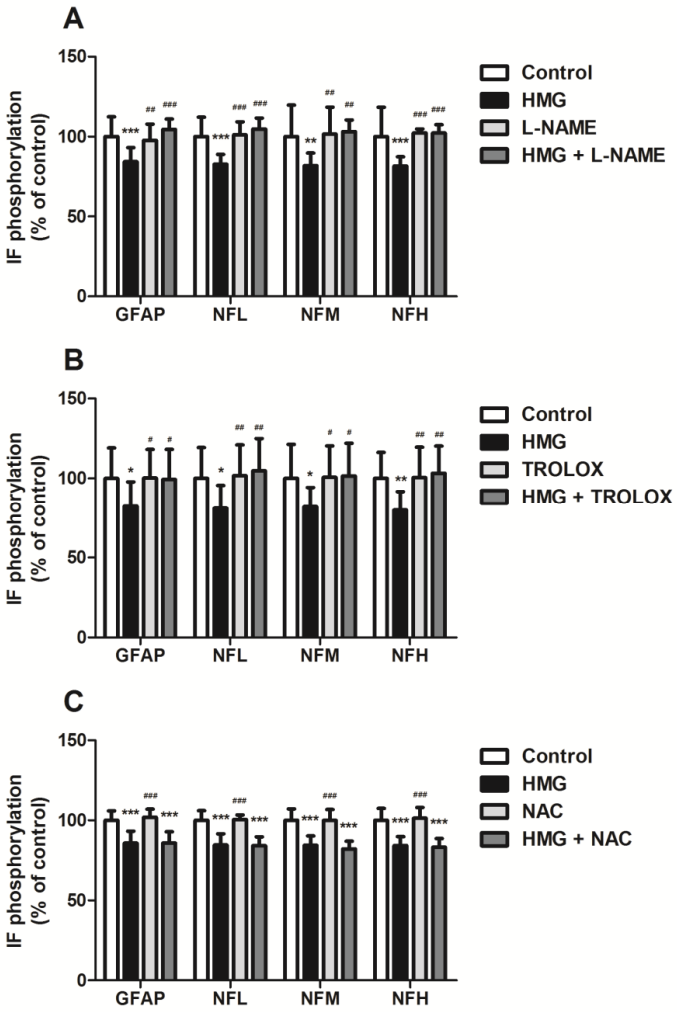


Figure 7 – Effects of L-NAME, TROLOX and NAC on HMG-induced alteration on the phosphorylation levels of intermediate filaments subunits (IFs) in the cytoskeletal fraction from striatal slices of 30-day-old rats. Tissue slices were co-incubated during 60 min in the presence or absence (control group) of the metabolite (5 mM) plus L-NAME, a nitric oxide synthase inhibitor (A;1 mM), TROLOX, a vitamin E hidrosoluble analogue (B;5 μM) or NAC, a GSH precursor (C;1 mM). Data are reported as mean ± SD of 18 animals in each group and expressed as percentage of controls. Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ compared with HMG.

Finally, we searched for whether L-NAME (1 mM) and TROLOX (5 μM) could prevent the inhibition of PKA and JNK activities provoked by HMG. Thus, striatal slices were co-incubated with HMG and TROLOX or L-NAME and the immunocontent

of the active enzyme subunits was quantified. Western blot analysis showed that TROLOX and L-NAME restored the immunocontent levels of the active form of PKA (Figure 8A and B) and JNK (Figure 8C and D), strengthening a role for oxidative/nitrosative stress on the altered phosphorylating system associated with the IF cytoskeleton of astrocytes and neurons in striatal slices treated with HMG.

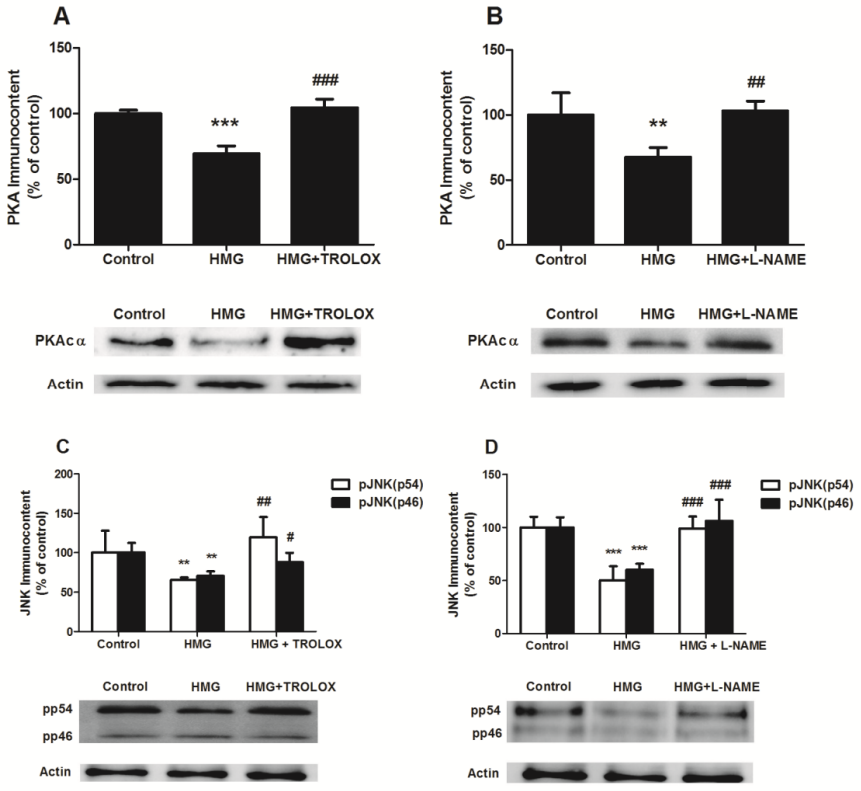


Figure 8 – Effects of TROLOX and L-NAME on PKAcα and JNK immunocontent in the total homogenate of striatal slices of 30-day-old rats. Tissue slices were incubated during 60 min in the presence or absence (control group) of the metabolite (5 mM) plus TROLOX (5 μM) or L-NAME (1 mM). Western blot with anti PKAcα (A and B); anti-pJNK (p54), anti-pJNK (p46) (C and D). Data are reported as mean ± SD of 6 animals in each group and expressed as percentage of controls. Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple range test. ** $p < 0.01$, *** $p < 0.001$ compared with control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ compared with HMG.

Discussion

In the present report, we described that HMG, MGA and MGT, at concentrations found to induce mitochondrial redox homeostasis dysfunction (Leipnitz et al, 2008a,b, 2009), were able to disrupt the cytoskeleton phosphorylating system of neural cells in striatal and cortical slices of 30-day-old rats. We initially demonstrated that these organic acids that accumulate in HMGA cause hypophosphorylation of GFAP and NF subunits (NFL, NFM and NFH). The data indicate for the first time that the phosphorylating system associated with the IF-enriched cytoskeleton of astrocytes and neurons is vulnerable to the effects of HMG, MGA and MGT and may contribute to the pathophysiological mechanisms of brain damage in this disease. It was also found that the hypophosphorylation induced by HMG was not due to energy deprivation, since important parameters of mitochondrial energy production were not altered by this compound. Similarly, HMG-elicited decrease of phosphorylated IF could not be attributed to activated protein phosphatases, since specific inhibitors of the most important Ser-Thr phosphatases known to be associated with these cytoskeleton (PP1, PP2A and PP2B) failed to prevent this effect. More interesting, we found that the hypophosphorylation provoked by HMG was mediated by down-regulated PKA and JNK/MAPK activities. The antioxidants TROLOX and the NOS inhibitor L-NAME normalized the phosphorylation levels of IFs and prevented the inhibition of PKA and JNK provoked by HMG in striatum, strongly indicating that oxidative and nitrosative damage of these protein kinases were responsible for the alterations observed. It is also conceivable that synaptic and extra-synaptic NMDA receptors may have also participated in these effects since the non-competitive NMDA antagonists MK-801and ifenprodil similarly restored the phosphorylated levels of IFs (Hardingham et al., 2002; Zhou et al., 2013a).

It is of note that striatum was used for elucidating the underlying mechanism of HMG, which most accumulates in HMGA, because progressive injury to the basal ganglia and usually found in patients with HMGA, attesting the vulnerability of this brain region. We also emphasize that leucoencephalopathy (cortical atrophy) is also detected in brain imaging of these individuals (van der Knaap et al., 1998; Yalçinkava et al., 1999; Yýlmaz et al., 2006). Furthermore, we have chosen to focus on the phosphorylating system associated with the IF cytoskeleton since these proteins are crucial for cell function, besides participating in the regulation of signaling pathways, axonal caliber and their dynamics is mostly regulated by phosphorylation (Sihag et al., 2007). Besides, several evidences point to a role for the IF cytoskeletal proteins in the cell response to an insult through alterations in the homeostasis of the phosphorylating system of these proteins (hyper and hypophosphorylation) observed in animal models of neurometabolic diseases (Pierozan et al., 2014; Loureiro et al., 2010a,b; Funchal et al., 2005a,b).

It is well known that hypophosphorylated NFs are more susceptible to proteolytic breakdown and accumulation of hypophosphoryltated NFs into the axons could disturb the axonal transport of organelles and intracellular compounds (Goldstein et al., 1987; Pant, 1988). In line with this, hypophosphorylated NF proteins have been associated with the neurotoxicity of quinolinic acid and tellurium and the consequent cell damage (Heimfarth et al., 2012a,b, 2013a,b; Pierozan et al., 2014). Also, IF hypophosphorylation in C6 glioma cells exposed to high homocysteine levels has been associated with actin reorganization and altered cell morphology (Loureiro et al., 2010a,b). Therefore, the effects observed in the present work let us suppose that *in vitro* changes on the phosphorylating system associated with the cytoskeleton can be a predictor of the potential *in vivo* neurotoxicity of HMG.

In our findings, decreased PKA activity was reflected by the hypophosphorylation of Ser55 in the amino-terminal head domain of NFL, which is implicated in the regulation of the polymerization/depolymerization dynamic of these proteins (Grant and Pant, 2000). In addition, decreased JNK activity was supported by hypophosphorylated Lys-Ser-Pro (KSP) repeats of NFM and NFH tail domain regions (Giasson and Mushynski, 1997). It is stressed that misregulated phosphorylation levels in NFM and NFH has long been considered to be associated with abnormal axonal transport rate and disrupted stability of mature axons (Sihag et al., 2007).

The relevance of NMDA-mediated actions of HMG on the phosphorylating mechanism associated with the cytoskeleton was also supported by Ca^{2+} buffering, which totally prevented IF hypophosphorylation. Our results corroborate previous evidence that induction of oxidative stress by HMG in intrastriatally injected rats is mediated by NMDA receptors (Fernandes et al., 2013). On the other hand, it is important to note that neither PKCaMII nor PP2B (two calcium-dependent enzymes) activities were altered in response to HMG, suggesting that IF hypophosphorylation donwstream of NMDA was not dependent exclusively on Ca^{2+} levels on the kinase/phosphatase activities, pointing to more complex mechanisms directed to the phosphorylating system.

Taking into account that activated GluN2BR gives rise to inhibition of cAMP/PKA/CREB signaling pathway (Corcoran et al., 2013) and that ifenprodil, a blocker of extra-synaptic NR2B subunit-containing NMDARs (Zhou et al., 2013a), prevented HMG-induced IF hypophosphorylation, we propose that PKA down-regulation could be, at least partially, ascribed to NR2B-mediated mechanisms of PKA inhibition. Our findings showing that IF proteins can be a target of NR2B actions are in line with the previously described role of NR2B in the regulation of hippocampal actin

cytoskeleton (Akashi et al., 2009). Also, GluN2B-containing NMDARs have been shown to have a role in the deleterious actions of oligomers of amyloid-beta peptide 1-42 in microtubule dynamics in hippocampal cultures, suggesting that NR2B could be implicated in cytoskeletal disruption and neuronal dysfunction (Mota et al., 2012). Interestingly, 3-hydroxyglutaric acid, a structurally HMG similar compound accumulated in glutaric academia type I, was shown to cause neuronal death by overactivation of the NR2B subunit of NMDA glutamate receptors (Kolker et al., 2000a,b), reinforcing the excitotoxic role of NR2B on brain metabolic pathology.

Regarding to the preventive effect of L-NAME on PKA and JNK activities, this is in line with the ability of NO to inhibit JNK via S-nitrosylation (Park et al., 2000). We could also presume that down-regulated JNK/MAPK may occur via co-activation of synaptic and extra-synaptic GluRs with induction of nNOS (Courtney et al., 2014; Zhou et al., 2013b). The observations that TROLOX, an effective scavenger of peroxyl radicals, normalized the reduction of protein kinases and the phosphorylating levels of IF provoked by HMG, may be tentatively associated with lipid peroxidation inducing these effects. This is in line with the previously reported modulation of the cytoskeleton in conditions of redox imbalance in different cell types and stress conditions (Loureiro et al., 2010a,b, 2013; Zamoner et al., 2008a,b).

We here demonstrated solid evidence for neurotoxic effects of the principal organic acids accumulating in HMGA disrupting cystoskeletal phosphorylating system with possible consequences for cellular function. We also found that antioxidants prevented these effects directed towards protein kinases, which is in accordance with previous findings showing that these metabolites disturb redox homeostasis (Leipnitz et al., 2008a,b, 2009; Fernandes et al., 2013; da Rosa et al., 2013). Therefore, it is conceivable that, besides hypoketotic hypoglycemia or hyperammonemia, the brain

damage in HMGA may be also a consequence of cytoskeleton dysregulation caused by high toxic levels of these organic acids.

In conclusion, this is the first report demonstrating that the disrupted homeostasis of the phosphorylating system associated with the cytoskeleton is caused by the major metabolites accumulating in HMGA. The evidence of a link between NMDA receptors, redox imbalance, misregulation of cell signaling mechanisms and hypophosphorylation of IF in response to HMG point to a critical role of the signaling pathways regulating the IF phosphorylation in the events of HMG-induced toxicity. Since disorganization of the cytoskeleton may be directly involved in several neurodegenerative disorders, we are tempted to propose that therapeutic strategies to regulate NMDA receptors and oxidative/nitrosative stress might serve as an adjuvant therapy for the protection of cells from the compounds that are found at high concentrations in this disease.

Conflicts of interest

The authors declare that there are no conflicts of interest associated with this manuscript.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, # 470236/2012-4), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, # 10/0031-1), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, # 2011/50400-0), Pró-Reitoria de Pesquisa/Universidade Federal do Rio Grande do Sul (PROPESQ/UFRGS, # PIBITI 18489), Rede Instituto Brasileiro de Neurociência (IBN-Net) (# 01.06.0842-00) and

Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção (INCT-EN, # 573677/2008-5).

References

Akashi, K., Kakizaki, T., Kamiya, H., Fukaya, M., Yamasaki, M., Abe, M., Natsume, R., Watanabe, M., Sakimura, K., 2009. NMDA receptor GluN2B (GluR epsilon 2/NR2B) subunit is crucial for channel function, postsynaptic macromolecular organization, and actin cytoskeleton at hippocampal CA3 synapses. J. Neurosci. 29, 10869-10882.

Akerman, K.E., Wikström, M.K., 1976. Safranine as a probe of the mitochondrial membrane potential. FEBS Lett. 68, 191-197.

Baba, Y., Hirukawa, N., Tanohira, N., Sodeoka, M., 2003. Structure-based design of a highly selective catalytic site-directed inhibitor of ser/Thr protein phosphatase 2B (calcineurin). J. Am. Chem. Soc 125, 740-749.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brownlees, J., Yates, A., Bajaj, N.P., Davis, D., Anderton, B.H., Leigh, P.N., Shaw, C.E., Miller, C.C., 2000. Phosphorylation of neurofilament heavy chain side-arms by stress activated protein kinase-1b/Jun N-terminal kinase-3. J. Cell Sci. 113, 401-407.

Cattani, D., Goulart, P.B., Cavalli, V.L., Winkelmann-Duarte, E., Dos Santos, A.Q., Pierozan, P., de Souza, D.F., Woehl, V.M., Fernandes, M.C., Silva, F.R., Gonçalves, C.A., Pessoa-Pureur, R., Zamoner, A., 2013. Congenital hypothyroidism alters the oxidative status, enzyme activities and morphological parameters in the hippocampus of developing rats. Mol. Cell. Endocrinol. 375, 14-26.

Corcoran, K.A., Leaderbrand, K., Radulovic, J., 2013. Extinction of Remotely Acquired Fear Depends on an Inhibitory NR2B/PKA Pathway in the Retrosplenial Cortex. J. Neurosci. 33, 19492-19498.

Courtney, M.J., Li, L.L., Lai, Y.Y., 2014. Mechanisms of NOS1AP action on NMDA receptor-nNOS signaling. Front. Cell. Neurosci. 8, 252.

da Rosa, M.S., Seminotti, B., Amaral, A.U., Fernandes, C.G., Gasparotto, J., Moreira, J.C., Gelain, D.P., Wajner, M., Leipnitz, G., 2013. Redox homeostasis is compromised in vivo by the metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency in rat cerebral cortex and liver. Free Radic. Res. 47, 1066-1075.

da Silva, C.G., Ribeiro, C.A., Leipnitz, G., Dutra-Filho, C.S., Wyse, A.T., Wannmacher, C.M., Sarkis, J.J., Jakobs, C., Wajner, M., 2002. Inhibition of cytochrome c oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid in vitro. Biochim. Biophys. Acta 1586, 81-91.

de Almeida, L.M., Funchal, C., Pelaez, P.deL., Pessutto, F.D., Loureiro, S.O., Vivian, L., Wajner, M., Pessoa-Pureur, R., 2003. Effect of propionic and methylmalonic acids on the in vitro phosphorylation of intermediate filaments from cerebral cortex of rats during development. Metab. Brain Dis. 18, 207-219.

de Mattos-Dutra, A., Sampaio de Freitas, M., Schröder, N., Fogaça Lisboa, C.S., Pessoa-Pureur, R., Wajner, M., 1997. In vitro phosphorylation of cytoskeletal proteins in the rat cerebral cortex is decreased by propionic acid. Exp. Neurol. 147, 238–247.

Faull, K., Bolton, P., Halpern, B., Hammond, J., Danks, D.M., Hähnel, R., Wilkinson, S.P., Wysocki, S.J., Masters, P.L., 1976. Letter: Patient with defect in leucine metabolism. N. Engl. J. Med. 294, 1013.

Fernandes, C.G., da Rosa, M.S., Seminotti, B., Pierozan, P., Martell, R.W., Lagranha, V.L., Busanello, E.N., Leipnitz, G., Wajner, M., 2013. In vivo experimental evidence that the major metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency induce oxidative stress in striatum of developing rats: a potential pathophysiological mechanism of striatal damage in this disorder. Mol. Genet. Metab. 109, 144-153.

Fischer, J.C., Ruitenbeek, W., Berden, J.A., Trijbels, J.M., Veerkamp, J.H., Stadhouders, A.M., Sengers, R.C., Janssen, A.J., 1985. Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin. Chim. Acta 153, 23-36.

Funchal, C., de Almeida, L.M., Oliveira Loureiro, S., Vivian, L., de Lima Pelaez, P., Dall Bello Pessutto, F., Rosa, A.M., Wajner, M., Pessoa Pureur, R., 2003. In vitro

phosphorylation of cytoskeleton proteins from cerebral cortex of rats. Brain Res. Brain Res. Protoc. 11, 111-118.

Funchal, C., Zamoner, A., dos Santos, A.Q., Loureiro, S.O., Wajner, M., Pessoa-Pureur, R., 2005a. Alpha-ketoisocaproic acid increases phosphorylation of intermediate filament proteins from rat cerebral cortex by mechanisms involving Ca^{2+} and cAMP. Neurochem. Res. 30, 1139-1146.

Funchal, C., Zamoner, A., dos Santos, A.Q., Moretto, M.B., Rocha, J.B., Wajner, M., Pessoa-Pureur, R., 2005b. Evidence that intracellular Ca^{2+} mediates the effect of alpha-ketoisocaproic acid on the phosphorylating system of cytoskeletal proteins from cerebral cortex of immature rats. J. Neurol. Sci. 238, 75-82.

Giasson, B.I., Mushynski, W.E., 1997. Study of proline-directed protein kinases involved in phosphorylation of the heavy neurofilament subunit. J. Neurosci, 17, 9466-9472.

Gibson, K.M., Breuer, J., Nyhan, W.L., 1988. 3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency: review of 18 reported patients. Eur. J. Pediatr. 148, 180-186.

Goldstein, M.E., Sternberger, N.H., Sternberger, L.A., 1987. Phosphorylation protects neurofilaments against proteolysis. J. Neuroimmunol. 14, 149–160.

Grant, P., Pant, H.C., 2000. Neurofilament protein synthesis and phosphorylation. J. Neurocytol. 29, 843-872.

Guidato, S., Bajaj, N.P., Miller, C.C., 1996. Cellular phosphorylation of neurofilament heavy-chain by cyclin-dependent kinase-5 masks the epitope for monoclonal antibody N52. Neurosci. Lett. 217, 157-160.

Hardingham, G.E., Fukunaga, Y., Bading, H., 2002. Extrasynaptic NMDARs oppose synaptic NMDArs by triggering BREB shut-off and cell death pathways. Nat. Neurosci. 5, 405-414.

Heimfarth, L., Loureiro, S.O., Reis, K.P., de Lima, B.O., Zamboni, F., Lacerda, S., Soska, A.K., Wild, L., da Rocha, J.B., Pessoa-Pureur, R., 2012a. Diphenyl ditelluride

induces hypophosphorylation of intermediate filaments through modulation of DARPP-32-dependent pathways in cerebral cortex of young rats. Arch. Toxicol. 86, 217-230.

Heimfarth, L., Loureiro, S.O., Dutra, M.F., Andrade, C., Pettenuzzo, L., Guma, F.T., Gonçalves, C.A., da Rocha, J.B., Pessoa-Pureur, R., 2012b. In vivo treatment with diphenyl ditelluride induces neurodegeneration in striatum of young rats: implications of MAPK and Akt pathways. Toxicol. Appl. Pharmacol. 264, 143-152.

Heimfarth, L., Loureiro, S.O., Dutra, M.F., Petenuzzo, L., de Lima, B.O., Fernandes, C.G., da Rocha, J.B., Pessoa-Pureur, R., 2013a. Disrupted cytoskeletal homeostasis, astrogliosis and apoptotic cell death in the cerebellum of preweaning rats injected with diphenyl ditelluride. Neurotoxicology 34, 175-188.

Heimfarth, L., Loureiro, S.O., Pierozan, P., de Lima, B.O., Reis, K.P., Torres, E.B., Pessoa-Pureur, R., 2013b. Methylglyoxal-induced cytotoxicity in neonatal rat brain: a role for oxidative stress and MAP kinases. Metab. Brain Dis. 28, 429-438.

Hisanaga, S., Gonda, Y., Inagaki, M., Ikai, A., Hirokawa, N., 1990. Effects of phosphorylation of the neurofilament L protein of filamentous structures. Cell Regul. 1, 237-248.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000a. Maturation-dependent neurotoxicity of 3-hydroxyglutaric and glutaric acids in vitro: a new pathophysiologic approach to glutaryl-CoA dehydrogenase deficiency. Pediatr. Res. 47, 495-503.

Kölker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2000b. Cerebral organic acids disorders induce damage via excitotoxic organic acids in vitro. Amino Acids 18, 31-40.

Kowaltowski, A.J., Castilho, R.F., Vercesi, A.E., 2001. Mitochondrial permeability transition and oxidative stress. FEBS Lett. 495, 12-15.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lai, J.C., Cooper, A.J., 1986. Brain alpha-ketoglutarate dehydrogenase complex: kinetic properties, regional distribution, and effects of inhibitors. J. Neurochem. 47, 1376-1386.

Leipnitz, G., Seminotti, B., Haubrich, J., Dalcin, K.B., Solano, A., de Bortoli, G., Rosa, R.B., Amaral, A.U., Dutra-Filho, C.S., Latini, A., Wajner, M., 2008a. Evidence that 3-hydroxy-3-mthylglutaric acid promotes lipid and protein oxidative damage and reduces the nonenzymatic antioxidant defenses in rat cerebral cortex. J. Neurosci. Res. 86, 683-693.

Leipnitz, G., Seminotti, B., Amaral, A.U., de Bortoli, G., Solano, A., Schuck, P.F., Wyse, A.T., Wannmacher, C.M., Latini, A., Wajner, M., 2008b. Induction of oxidative stress by the metabolites accumulating in 3-methylglutaconic aciduria in cerebral cortex of young rats. Life Sci. 82, 652-662.

Leipnitz, G., Seminotti, B., Fernandes, C.G., Amaral, A.U., Beskow, A.P., da Silva, L.deB., Zanatta, A., Ribeiro, C.A., Vargas, C.R., Wajner, M., 2009. Striatum is more vulnerable to oxidative damage induced by the metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency as compared to liver. Int. J. Dev. Neurosci. 27, 351-356.

Liu Y, Peterson DA, Kimura H, Schubert D, 1997. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. J Neurochem 69:581-93.

Loureiro, S.O., Heimfarth, L., Lacerda, B.A., Vidal, L.F., Soska, A., dos Santos, N.G., de Souza Wyse, A.T., Pessoa-Pureur, R., 2010a. Homocysteine induces hypophosphorylation of intermediate filaments and reorganization of actin cytoskeleton in C6 glioma cells. Cell. Mol. Neurobiol. 30, 557-568.

Loureiro, S.O., Romão, L., Alves, T., Fonseca, A., Heimfarth, L., Moura Neto, V., Wyse, A.T., Pessoa-Pureur, R., 2010b. Homocysteine induces cytoskeletal remodeling and production of reactive oxygen species in cultured cortical astrocytes. Brain Res. 1355, 151-164.

Loureiro, S.O., Heimfarth, L., Scherer, E.B., da Cunha, M.J., de Lima, B.O., Biasibetti, H., Pessoa-Pureur, R., Wyse, A.T., 2013. Cytoskeleton of cortical astrocytes as a target to proline through oxidative stress mechanisms. Exp. Cell Res. 319, 89-104.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Middeldorp, J., Hol, E.M., 2011. GFAP in health and disease. Prog. Neurobiol. 93, 421-443.

Mirandola, S.R., Melo, D.R., Schuck, P.F., Ferreira, G.C., Wajner, M., Castilho, R.F., 2008. Methylmalonate inhibits succinate-supported oxygen consumption by interfering with mitochondrial succinate uptake. J. Inherit. Metab. Dis. 31, 44-54.

Morrison, J.F., 1954. The activation of aconitase by ferrous ions and reducing agents. Biochem. J. 58, 685-692.

Mota, S.I., Ferreira, I.L., Pereira, C., Oliveira, C.R., Rego, A.C., 2012. Amyloid-beta peptide 1-42 causes microtubule deregulation through N-methyl-D-aspartate receptors in mature hippocampal cultures. Curr. Alzheimer Res. 9, 844-856.

Pant, H.C., 1988. Dephosphorylation of neurofilament proteins enhances their susceptibility to degradation by calpain. Biochem. J. 256, 665-668.

Park, H.S., Huh, S.H., Kim, M.S., Lee, S.H., Choi, E.J., 2000. Nitric oxide negatively regulates c-JunN-terminalkinase/stress-activated protein kinase by means of S-nitrosylation. Proc. Natl. Acad. Sci. USA 97, 14382-14387.

Perrot, R., Eyer, J., 2009. Neuronal intermediate filaments and neurodegenerative disorders. Brain Res. Bull. 80, 282-295.

Pié, J., López-Viñas, E., Puisac, B., Menao, S., Pie, A., Casale, C., Ramos, F.J., Hegardt, F.G., Gómez-Puertas, P., Casals, N., 2007. Molecular genetics of HMG-CoA lyase deficiency. Mol. Genet. Metab. 92, 198-229.

Pierozan, P., Zamoner, A., Soska, A.K., Silvestrin, R.B., Loureiro, S.O., Heimfarth, L., Mello e Souza, T., Wajner, M., Pessoa-Pureur, R., 2010. Acute intrastriatal

administration of quinolinic acid provokes hyperphosphorylation of cytoskeletal intermediate filament proteins in astrocytes and neurons of rats. Exp. Neurol. 224, 188-196.

Pierozan, P., Zamoner, A., Soska, Â.K., de Lima, B.O., Reis, K.P., Zamboni, F., Wajner, M., Pessoa-Pureur, R., 2012. Signaling mechanisms downstream of quinolinic acid targeting the cytoskeleton of rat striatal neurons and astrocytes. Exp. Neurol. 233, 391-399.

Pierozan, P., Gonçalves Fernandes, C., Ferreira, F., Pessoa-Pureur, R., 2014. Acute intrastriatal injection of quinolinic acid provokes long-lasting misregulation of the cytoskeleton in the striatum, cerebral cortex and hippocampus of young rats. Brain Res. 1577, 1-10.

Reimão, S., Morgado, C., Almeida, I.T., Silva, M., Corte Real, H., Campos, J., 2009. 3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency: initial presentation in a young adult. J. Inherit. Metab. Dis. 32, S49-S52.

Reis de Assis, D., Maria, R.deC., Borba Rosa, R., Schuck, P.F., Ribeiro, C.A., da Costa Ferreira, G., Dutra-Filho, C.S., Terezinha de Souza Wyse, A., Duval Wannmacher, C.M., Santos Perry, M.L., Wajner, M., 2004. Inhibition of energy metabolism in cerebral cortex of young rats by the medium-chain fatty acids accumulating in MCAD deficiency. Brain Res. 1030, 141-151.

Rittase, W.B., Dong, Y., Barksdale, D., Galdzicki, Z., Bausch, S.B., 2014. Dynorphin up-regulation in the dentate granule cell mossy fiber pathway following chronic inhibition of GluN2B-containing NMDAR is associated with increased CREB (Ser 133) phosphorylation, but is independent of BDNF/TrkB signaling pathways. Mol. Cell. Neurosci. 60, 63-71.

Rosenthal, R.E., Hamud, F., Fiskum, G., Varghese, P.J., Sharpe, S., 1987. Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. J. Cereb. Blood Flow Metab. 7, 752-758.

Rustin, P., Chretien, D., Bourgeron, T., Gerard, B., Rotig, A., Saudubray, J.M., Munnich, A., 1994. Biochemical and molecular investigations in respiratory chain deficiencies. Clin. Chim. Acta 228, 35-51.

Sheppeck, J.E., Gauss, C.M., Chamberlin, A.R., 1997. Inhibition of the Ser-Thr phosphatases PP1 and PP2A by naturally occurring toxins. Bioorg. Med. Chem. 5, 1739-1750.

Sihag, R.K., Nixon, R.A., 1989. In vivo phosphorylation of distinct domains of the 70-kilodalton neurofilament subunit involves different protein kinases. J. Biol. Chem. 264, 457-464.

Sihag, R.K., Inagaki, M., Yamaguchi, T., Shea, T.B., Pant, H.C., 2007. Role of phosphorylation on the structural dynamics and function of types III and IV intermediate filaments. Exp. Cell Res. 313, 2098-2109.

Strack, S., Westphal, R.S., Colbran, R.J., Ebner, F.F., Wadzinski, B.E., 1997. Protein serine/threonine phosphatase 1 and 2A associate with and dephosphorylate neurofilaments. Brain Res. Mol. Brain Res. 49, 15-28.

Sweetman, L., Wlliams, J.C., 2001. Branched chain organic acidurias, in: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp. 2340-2342.

Tretter, L., Adam-Vizi, V., 2000. Inhibition of Krebs cycle enzymes by hydrogen peroxide: A key role of [alpha]-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress. J. Neurosci. 20, 8972-8979.

van der Knaap, M.S., Bakker, H.D., Valk, J., 1998. MR imaging and proton spectroscopy in 3-hydroxy-3-methylglutaryl coenzyme A lyase deficiency. AJNR Am. J. Neuroradiol. 19, 378-382.

Veeranna, G.J., Amin, N.D., Ahn, N.G., Jaffe, H., Winters, C.A., Grant, P., Pant, H.C., 1998. Mitogen-activated protein kinases (Erk1,2) phosphorylate lys-ser-pro (KSP) repeats in neurofilament proteins NF-H and NF-M. J. Neurosci. 18, 4008-4021.

Veeranna, G.J., Shetty, K.T., Takahashi, M., Grant, P., Pant, H.C., 2000. Cdk5 and MAPK are associated with complexes of cytoskeletal proteins in rat brain. Brain Res. Mol. Brain Res. 76, 229-236.

Viegas, C.M., Zanatta, Â., Grings, M., Hickmann, F.H., Monteiro, W.O., Soares, L.E., Sitta, Â., Leipnitz, G., de Oliveira, F.H., Wajner, M., 2014. Disruption of redox homeostasis and brain damage caused in vivo by methylmalonic acid and ammonia in cerebral cortex and striatum of developing rats. Free Radic. Res. 48, 659-669.

Whitaker, A.N., McKay, D.G., 1969. Studies of catecholamine shock. I. Disseminated intravascular coagulation. Am. J. Pathol. 65, 153-176.

Wysocki, S.J., Hähnel, R., 1986. 3-hydroxy-3-methylglutaryl-coenzyme A lyase deficiency: a review. J. Inherit. Metab. Dis. 9, 225-233.

Yalçinkaya, C., Dinçer, A., Gündüz, E., Fiçicioğlu, C., Koçer, N., Aydin, A., 1999. MRI and MRS in HMG-CoA lyase deficiency. Pediatr. Neurol. 20, 375-380.

Yýlmaz, Y., Ozdemir, N., Ekinci, G., Baykal, T., Kocaman, C., 2006. Corticospinal tract involvement in a patient with 3-HMG coenzyme A lyase deficiency. Pediatr. Neurol. 35, 139-141.

Zafeiriou, D.I., Augoustides-Savvopoulou, P., Haas, D., Smet, J., Triantafyllou, P., Vargiami, E., Tamiolaki, M., Gombakis, N., van Coster, R., Sewell, A.C., Vianey-Saban, C., Gregersen, N., 2007. Ethylmalonic encephalopathy: clinical and biochemical observations. Neuropediatrics 38, 78-82.

Zamoner, A., Pierozan, P., Vidal, L.F., Lacerda, B.A., Dos Santos, N.G., Vanzin, C.S., Pessoa-Pureur, R., 2008a. Vimentin phosphorylation as a target of cell signaling mechanisms induced by 1α,25-dihydroxyvitamin D3 in immature rat testes. Steroids 73, 1400-1408.

Zamoner, A., Barreto, K.P., Filho, D.W., Sell, F., Woehl, V.M., Guma, F.C., Pessoa-Pureur, R., Silva, F.R., 2008b. Propylthiouracil-induced congenital hypothyroidism upregulates vimentin phosphorylation and depletes antioxidant defenses in immature rat testis. J. Mol. Endocrinol. 40, 125-135.

Zhou, X., Ding, Q., Chen, Z., Yun, H., Wang, H., 2013a. Involvement of the GluN2A and GluN2B subunits in synaptic and extrasynaptic N-methyl-D-aspartate receptor function and neuronal excitotoxicity. J. Biol, Chem. 288, 24151-24159.

Zhou, X., Hollern, D., Liao, J., Andrechek, E., Wang, H., 2013b. NMDA receptor-mediated excitotoxicity depends on the coactivation of synaptic and extrasynaptic receptors. Cell Death Dis. 4, e560.

PARTE III

Discussão e Conclusões

III DISCUSSÃO

A HMGA é um distúrbio genético de caráter autossômico recessivo com prevalência estimada de 1 para cada 100.000 nascimentos (PIÉ *et al*., 2007; REIMÃO *et al*., 2009). É causada pela deficiência da HL, resultando em um acúmulo tecidual e uma alta excreção urinária dos ácidos 3-hidroxi-3-metilglutárico (HMG), 3-metilglutárico (MGA) e 3-metilglutacônico (MGT), bem como em menor grau dos ácidos 3-hidroxi-isovalérico e 3-metilcrotonilglicina nos indivíduos afetados (BONAFÉ *et al*., 2000; SWEETAMN e WILLIAMS, 2001; PIÉ *et al*., 2007). A apresentação clínica geralmente ocorre durante o primeiro ano de vida, usualmente após um período de jejum prolongado ou outro evento catabólico. Os episódios agudos são comuns e são caracterizados por vômitos, diarréia, desidratação, hipotonia, hipotermia, letargia, coma e apnéia, bem como por acidose metabólica e hipoglicemia hipocetótica. Outros sinais incluem macrocefalia, atraso no desenvolvimento, hepatomegalia com alterações no exame de fígado, pancreatite aguda e cardiomiopatia dilatada (SWEETMAN e WILLIAMS, 2001; GIBSON *et al*., 1994; FUNGHINI *et al*., 2001; VARGAS *et al*., 2007; ZAFEIRIOU *et al*., 2007). Esta doença é fatal em aproximadamente 20% dos casos, embora os sintomas tendem a ser mais leves depois da infância (THOMPSON *et al*., 1990; PIÉ *et al*., 2003).

Praticamente todos os pacientes apresentam sintomas neurológicos graves e anormalidades no córtex cerebral e gânglios da base (YALÇINKAYA *et al*., 1999; YÝLMAZ *et al*., 2006; ZAFEIRIOU *et al*., 2007) cuja fisiopatogenia é pouco conhecida. Entretanto, podemos presumir que o acúmulo crônico desses metabólitos pode representar, ao menos em parte, um mecanismo

importante que contribui para os sintomas e anormalidades cerebrais observadas nestes pacientes, especialmente durante episódios de descompensação metabólica, em que as concentrações dos ácidos orgânicos aumentam drasticamente. Portanto, no presente estudo investigamos os efeitos dos principais metabólitos acumulados na HMGA sobre importantes parâmetros de estresse oxidativo, sobre a resposta inflamatória, bem como sobre a fosforilação de proteínas do citoesqueleto no estriado e córtex cerebral de ratos, com o objetivo de esclarecer a relação entre as características clínicas e as alterações bioquímicas nessa doença. É digno de nota que o estriado foi utilizado porque nessa doença ocorre lesão progressiva nos gânglios da base (estriado nos roedores), atestando a vulnerabilidade dessa região do cérebro. Ressaltamos, ainda, que a leucoencefalopatia (atrofia cortical) também é comumente detectada em imagens do cérebro desses indivíduos, justificando o uso do córtex cerebral neste estudo (van der KNAAP *et al.*,1998; YALÇINKAYA *et al.*, 1999; YÝLMAZ *et al.*, 2006).

Como já mencionado, os mecanismos subjacentes da patogênese das lesões cerebrais na HMGA são pouco conhecidos. No entanto, tem sido sugerido que além do acúmulo intracelular de ácidos orgânicos tóxicos, hipoglicemia associada com déficit de corpos cetônicos, deficiência de carnitina podem também estar envolvidos na patofisiologia da doença (KAHLER *et al.*, 1994; MITCHELL e FUKAO, 2001; LEUNG *et al.*, 2009). Neste contexto, estudos *in vitro* anteriores demonstraram que o HMG e o MGA induzem dano oxidativo lipídico e protéico e reduzem as defesas antioxidantes não enzimáticas no córtex cerebral, no estriado e no fígado de ratos jovens (LEIPNITZ *et al.*, 2008a,b e 2009). Mostrou-se também que o MGA induz

disfunção mitocondrial e inibe a atividade da Na +, K + -ATPase em sinaptossomas preparados a partir de cérebro de ratos (RIBEIRO *et al.*, 2011).

A partir desses achados iniciamos nossa investigação estudando os efeitos *ex vivo* da administração intrastriatal de HMG e MGA sobre parâmetros de estresse oxidativo em estriado de ratos em desenvolvimento (Capítulo 1). Avaliamos se altas concentrações de HMG e MGA no estriado de ratos jovens obtidas por uma injeção intrastriatal aguda desses ácidos orgânicos poderiam afetar a homeostase redox na esperança de esclarecer melhor os mecanismos patológicos envolvidos nas anormalidades do estriado, uma estrutura cerebral alterada no cérebro dos pacientes afetados (van der KNAAP *et al.*,1998; YALÇINKAYA *et al.*, 1999; YÝLMAZ *et al.*, 2006). Para isso, foram determinados parâmetros importantes de estresse oxidativo, incluindo as concentrações de MDA (peroxidação lipídica), a formação de carbonilas (dano oxidativo protéico), a produção de espécies reativas (oxidação da DCFH e produção de óxido nítrico), as concentrações de GSH (defesas antioxidantes não enzimáticas) e as atividades das enzimas antioxidantes SOD, GR, G6PD, GPx e CAT.

Nossos resultados demonstram que o HMG e o MGA induziram dano oxidativo a lipídios e a proteínas, bem como aumentaram a produção de espécies reativas de oxigênio, enquanto o HMG também induziu a produção de espécies reativas de nitrogênio. Em relação às defesas antioxidantes, ambos os metabólitos diminuíram os níveis de GSH e causaram alterações em várias atividades enzimáticas, provocando um desequilíbrio nesses sistemas de defesa. Finalmente observou-se que os antioxidantes melatonina, N-

acetilcisteína e a combinação de vitaminas C e E preveniram completamente ou atenuaram as alterações dos parâmetros de estresse oxidativo causadas pelo HMG, reforçando a participação de espécies reativas, mais particularmente os radicais hidroxila e peroxila que são sequestrados por estes antioxidantes (REITER *et al.*, 2001; ANISIMOV, 2006; HALLIWELL e GUTERIDGE 2007). Verificamos também que o MK-801, um antagonista não-competitivo do receptor glutamatérgico do tipo NMDA, impediu alguns dos efeitos provocados pelo HMG, indicando o envolvimento dos receptores NMDA nos efeitos encontrados. O fato de receptores NMDA estarem envolvidos nos efeitos induzidos por HMG não é surpreendente, devido à semelhança estrutural entre a HMG e glutamato. Portanto, pode ser de interesse estudar se os ácidos orgânicos acumulados e, especialmente, o HMG interagem diretamente com receptores NMDA. Por outro lado, não se pode excluir a possibilidade de o aumento na produção das ERN pode ser mediada por uma indução da atividade da óxido nítrico sintase, que conduz à geração dessas espécies foi, pelo menos, parcialmente devido a supraestimulação dos receptores NMDA por este ácido orgânico (HALLIWELL e GUTTERIDGE, 2007; BROWN, 2010; AYATA *et al.*, 1997).

Nossos resultados indicam fortemente que o HMG e o MGA provocam estresse oxidativo in vivo em estriado de ratos jovens. Devemos salientar também que as espécies reativas são capazes de oxidar moléculas diferentes, incluindo lípidos, proteínas, açúcares e DNA, o que representa um evento chave na cascata que conduz à patogenicidade da morte celular necrótica ou apoptótica (KROEMER e REED, 2000; NIZUMA *et al.*, 2009; CIRCU e AW, 2010). Além disso, o estresse oxidativo é uma condição muito prejudicial,

especialmente para o cérebro em comparação com outros tecidos, devido ao fato de o cérebro possuir uma alta taxa de metabolismo oxidativo acoplado a uma grande produção de ERO, uma atividade reduzida das enzimas antioxidantes, menores concentrações de antioxidantes e maior potencial de peroxidação, devido ao seu elevado teor de PUFA (HALLIWELL e GUTTERIDGE, 2007).

A partir do conhecimento de que o cérebro é bastante suscetível ao estresse oxidativo causado pelo HMG e pelo MGA fomos investigar se os astrócitos são células alvo desses metabólitos, já que essas células neurais são fundamentias para o funcionamento do sistema nervoso central e susceptíveis a danos por neurotoxinas, além de serem importantes fontes de produção de espécies reativas. Para tal determinamos os efeitos do HMG e MGA sobre importantes parâmetros da homeostase redox, produção de citocinas, bem como sobre a função mitocondrial e a viabilidade de astrócitos cultivados obtidos de córtex cerebral de ratos neonatos (Capítulo 2). Descobrimos que ambos os ácidos orgânicos reduziram a função mitocondrial astrocitária, sem alterar a viabilidade celular, reduzindo também as concentrações de GSH. Em contrapartida, os metabólitos aumentaram a formação de espécies reativas verificada por um aumento na oxidação da DCFH e provocaram um aumento significativo das citocinas pró-inflamatórias IL-1β, IL-6 e TNFα através da via de sinalização da Erk.

Assim, reforçando nossos resultados encontrados no capítulo 1, verificamos que o HMG e o MGA provocaram um aumento na produção de espécies reativas. Uma vez que a oxidação da DCFH ocorre principalmente por

meio de ERO e é utilizada como uma sonda para a determinação de particularmente ERO em sistemas biológicos, concluiu-se que estes ácidos orgânicos provocaram a geração de espécies reativas em um sistema de células intactas (astrócitos), corroborando com resultados anteriores (LEIPNITZ *et al*., 2008a,b e 2009; RIBEIRO *et al*., 2011). Não podemos, no entanto excluir a participação de ERN nesse modelo, visto que a oxidação da DCFH pode também ocorrer através dessas espécies, assim como citado no capítulo 1 deste trabalho. Tais resultados indicam que os astrócitos expostos aos principais metabólitos que se acumulam na HMGA estão envolvidos na produção de radicais livres.

Descobrimos também que estes ácidos orgânicos diminuíram marcadamente as concentrações de GSH. Nesse contexto, os astrócitos são considerados uma das principais fontes de GSH por sintetizar e secretar esse antioxidante, ajudando, assim, a manutenção do estado redox neuronal (DRINGEN, 2000; POPE *et al*., 2008). GSH é o antioxidante mais abundante no cérebro, exercendo um papel fundamental para eliminar as ERO, proteger os grupamentos sulfidrila das proteínas no estado redox adequado, regulando também a morte celular e as cascatas de sobrevivência celular (SARAFIAN *et al*., 1996).

Uma vez que níveis adequados de antioxidantes são essenciais para proteger as células contra os danos oxidativos (CASTOLDI *et al*., 2001), é possível que a redução significativa da GSH induzida pelos metabólitos que se acumulam na HMGA pode causar perda da funcionalidade de astrócitos expostos ao HMG e ao MGA, como observado pela redução significativa de

MTT. Salienta-se que o ensaio MTT é comumente utilizado para avaliar a funcionalidade mitocondrial, como um indicador do estado energético das células refletindo a atividade de desidrogenases, embora também seja aceito como um marcador de viabilidade celular. Uma vez que a incorporação de iodeto de propídio não foi alterado em astrócitos em cultura expostos ao HMG e ao MGA nas mesmas condições, conclui-se que a viabilidade celular foi preservada (TSAI e LEE, 1994; ALARCÓN-AGUILAR *et al.*, 2014).

Não podemos estabelecer no presente se a redução dos níveis de GSH ocorreu devido à sua oxidação por radicais livres devido a uma alta produção de espécies reativas (aumento da oxidação DCFH) causada pelos ácidos orgânicos que leva ao consumo de GSH, ou se a redução dos níveis de GSH levou a um desequilíbrio na formação de espécies reativas levando a um estado pró-oxidante. No entanto, enfatiza-se que a depleção de GSH ocorre durante condições de estresse que podem ser consequência de uma resposta inflamatória com estresse oxidativo, desempenhando portanto um papel crítico na resposta inflamatória astrocítica (TANABE *et al.*, 2010; LEE *et al.*, 2010).

Nesse particular, uma contribuição original de nossa investigação (capítulo 2) foi a demonstração de níveis elevados de citocinas IL-1β, IL-6 e TNFα em astrócitos cultivados expostos a HMG e MGA. TNFα e IL-1β são sintetizadas predominantemente por microglia e astrócitos na resposta inflamatória aguda. Essas citocinas também podem induzir a síntese de IL-6. A IL-6 é produzida em microglia, astrócitos e neurônios e desempenha um papel central numa variedade de funções do sistema nervoso central, tais como a indução e a modulação de ativação de astrócitos, inflamação e neuroproteção

(TANABE *et al*., 2010; LEE *et al*., 2010; SANTELLO *et al*., 2011; ERTA *et al*., 2012; YE *et al*., 2013). Além disso, a elevação dos níveis de citocinas provavelmente reflete a reatividade da glia que, em combinação com um aumento da produção de ER e um decréscimo no teor de antioxidantes no cérebro, foram descritas como envolvidas na patogênese e no desenvolvimento de várias doenças humanas e na neurodegeneração (DARLINGTON, 2005; KANWAR e NEHRU, 2007).

Assim, verificamos que o HMG e o MGA induziram as citocinas pró-inflamatórias clássicas (TNFα e IL1-β) dependentes da via de sinalização Erk. No que diz respeito ao IL-6, esta citocina apresenta uma dualidade de ações, degenerativas e reparadoras, anti- e pró-inflamatórias (FARINA *et al*., 2007) Neste particular, é de se notar que a via Erk tem sido implicada na regulação da resposta inflamatória glial após um insulto (dano) e é uma via de transdução de NFκB (LEE *et al*., 2010; SANTELLO *et al*., 2011; ERTA *et al*., 2012; YE *et al*., 2013; DARLINGTON, 2005; KANWAR e NEHRU, 2007; BRENNER, 2014; BOBERMIN *et al*., 2012). NFκB é considerado o principal mediador inflamatório no SNC, embora o seu mecanismo de ativação e subsequente translocação para o núcleo não são completamente compreendidos (GLOIRE *et al*., 2006; WAKABAYASHI *et al*., 2010). Por outro lado, o aumento de IL-6 não foi associado com a via da Erk, mas pode ter sido causado alternativamente pelo aumento de TNFα e IL1-β, indutores de IL-6 (TANABE *et al*., 2010; TANABE *et al*., 2011).

Os resultados obtidos no capítulo 2 indicam uma susceptibilidade de astrócitos corticais de ratos neonatos aos insultos tóxicos do HMG e do MGA,

associado a um aumento da resposta pró-inflamatória com aumento da produção de citocinas e de estresse oxidativo acompanhada por uma disfunção mitocondrial. Esses resultados confirmam nossos achados de indução de estresse oxidativo em estriado *in vivo* por esses compostos, bem como achados anteriores *in vitro* realizados em frações subcelulares e homogeneizados de cérebro (LEIPNITZ *et al*., 2008a,b; 2009) e indicam que a perturbação da homeostase redox astrocitária causada pelos principais ácidos orgânicos que se acumulam na HMGA pode potencialmente contribuir para a neuropatologia de pacientes afetados por esta doença. Além disso, nossos dados indicam que astrócitos em cultura representam uma ferramenta biológica interessante e promissora para estudar a toxicidade dos compostos que se acumulam em outras doenças hereditárias metabólicas, embora a sua principal limitação dos achados presentes reside no fato de que não envolve as relações complexas entre neurônios e células gliais. Presume-se, portanto, que a produção de espécies reativas e citocinas contribuem para a disfunção neurológica observada nesta doença, particularmente durante as crises metabólicas, em que a produção e o acúmulo dos metabólitos no cérebro provavelmente é ainda maior. No entanto, não se pode descartar que a hiperamonemia e a privação de energia (hipoglicemia hipocetótica) também podem estar envolvidas na lesão cerebral desses pacientes uma vez que estas condições também podem resultar na formação de radicais livres no SNC (BOBERMIN *et al*., 2012; SINGH *et al*., 2004; SUH *et al*., 2007).

Finalmente investigamos os efeitos do HMG, MGA e MGT sobre a fosforilação da GFAP e das subunidades dos NF (NFL, NFM e NFH) do citoesqueleto em fatias de estriado e córtex cerebral de ratos jovens (Capítulo

3). Optamos por focar nosso estudo no sistema de fosforilação associada aos FI do citoesqueleto, porque essas proteínas são cruciais para a morfologia celular, além de participarem da regulação de vias de sinalização, na formação e manutenção do calibre axonal e também porque essas dinâmicas são principalmente reguladas por fosforilação (SIHAG *et al*., 2007) . Além disso, várias evidências apontam para um papel dos FI na resposta a um insulto celular através de alterações na homeostase do sistema de fosforilação dessas proteínas (hiper e hipofosforilação) observada em modelos animais de doenças neurometabólicas (PIEROZAN *et al*., 2014; LOUREIRO *et al*., 2010a,b; FUNCHAL *et al*., 2005a,b).

Resumidamente, nossos resultados obtidos no capítulo 3 foram que o HMG, o MGA e o MGT provocaram uma hipofosforilação da GFAP e das subunidades dos NF nas duas estruturas cerebrais testadas e esse efeito foi mediado pela inibição das proteínas cinases PKA e JNK, sem a participação das proteínas fosfatases. Observou-se também que a hipofosforilação do citoesqueleto era dependente dos receptores do tipo NMDA sinápticos e extrasinápticos, bem como do íon Ca^{2+}. Além disso, os antioxidantes TROLOX e L-NAME preveniram completamente a hipofosforilação e a inibição das atividades da PKA e da JNK causadas pelo HMG.

Esses dados indicam que o sistema de fosforilação do citoesqueleto associado com os FI de astrócitos e neurônios é vulnerável aos efeitos do HMG, do MGA e do MGT, podendo contribuir para explicar os mecanismos patofisiológicos de dano cerebral dos pacientes com a deficiência da HL. Verificou-se também que o hipofosforilação induzida pelo HMG não foi devido

ao déficit energético (o ATP é necessário para que haja a correta fosforilação de proteínas), uma vez que parâmetros importantes da produção de energia não foram alteradas pelo HMG. Também demonstramos que a diminuição da fosforilação dessas proteínas não poderia ser atribuída a ativação de proteínas fosfatases, uma vez que os inibidores específicos Ser-Thr das fosfatases mais importantes associados com o citoesqueleto (PP1, PP2A e PP2B) não conseguiram evitar este efeito. Contudo, descobrimos que a hipofosforilação provocada pelo HMG foi mediada pela inibição das proteínas cinases PKA e JNK. Além disso, observamos que os antioxidantes TROLOX e o inibidor da óxido nitrico sintase (NOS), L-NAME, preveniram totalmente a hipofosforilação dos FI e a inibição da atividade da PKA e da JNK provocadas pelo HMG no estriado, indicando fortemente que ERO e ERN poderiam ter provocado dano oxidativo a essas proteínas cinases e serem responsáveis pelas alterações de fosforilação observadas. Também é concebível que os receptores NMDA sinápticos e extra-sinápticos também podem ter participado nesses efeitos uma vez que os antagonistas de NMDA não competitivo, MK-801, e seletivo da subunidade NR2B, ifenprodil, normalizaram os níveis de fosforilação dos FI (HARDINGHAM *et al*., 2002; ZHOU *et al*., 2013a,b).

Para reforçar a participação das cinases nos efeitos causados por HMG verificamos que a diminuição da atividade da PKA foi refletida pela hipofosforilação da Ser55 no domínio aminoterminal da NFL que está implicado na regulação da dinâmica de polimerização / despolimerização dessas proteínas (GRANT e PANT, 2000). Além disso, a diminuição da atividade da JNK refletiu-se na hipofosforilação das repetições do tipo KSP nos domínios carboxiterminal do NFH e do NFN (GIASSON e MUSHYNSKI, 1997). Ressalte-

se que os níveis de fosforilação do NFM e do NFH tem sido associados com a velocidade de transporte axonal e a estabilidade dos axônios maduros (SIHAG *et al*., 2007).

Os efeitos do HMG sobre o mecanismo de fosforilação associada ao citoesqueleto mediados por NMDA foi reforçada pela importância do Ca^{2+} nesses efeitos, já que o tamponamento e o sequestro desse íon totalmente preveniram essa hipofosforilação. Esses resultados corroboram com os resultados encontrados no capítulo 1 desta tese de que a indução de estresse oxidativo causado por injeção intracerebral de HMG é mediada por receptores glutamatérgicos do tipo NMDA.

Levando-se em conta que a ativação de receptores glutamatérgicos que contém a subunidade NR2B leva à inibição da sinalização CREB via AMPc / PKA (CORCORAN *et al*., 2013) e que o ifenprodil, um bloqueador seletivo dessa subunidade extrasináptica (ZHOU *et al*., 2013a,b) preveniu a hipofosforilação dos FI provocada por HMG, é possível que esse efeito tenha sido mediado pela inibição da PKA secundariamente à ativação dos receptores NMDA extrasinápticos. Nossos resultados mostram que proteínas do citoesqueleto podem ser alvo de ações indiretas ao estímulo dos NR2B, o que está de acordo com o papel descrito anteriormente desses receptores na regulação do citoesqueleto de actina no hipocampo (AKASHI *et al*., 2009). Além disso, os NR2B têm demostrado possuir um papel prejudicial na dinâmica dos microtúbulos em culturas de hipocampo com consequente disfunção neuronal (MOTA *et al*., 2012). Curiosamente, o ácido 3-hidroxiglutárico, um composto estruturalmente semelhante ao HMG, que se acumula na acidemia

glutárica tipo I, foi demonstrado provocar morte neuronal por superativação da subunidade NR2B de receptores NMDA (KÖLKER *et al*., 2000a,b), reforçando portanto o papel excitotóxico dessa subunidade NMDA no cérebro de doenças metabólicas.

Assim, no capítulo 3, demonstramos evidências de que os principais ácidos orgânicos que se acumulam na HMGA alteram o sistema de fosforilação do citoesqueleto com possíveis consequências para a forma e função celular. Descobrimos também que alguns antioxidantes impediram esses efeitos inibitórios em proteínas cinases, o que está de acordo com achados prévios (LEIPNITZ *et al*., 2008a,b e 2009; da ROSA *et al*., 2013), além de reforçar os resultados encontrados nos capítulos anteriores desta tese, mostrando que esses metabólitos perturbam a homeostase redox no cérebro. Portanto, é concebível que, além da hipoglicemia-hipocetótica e da hiperamonemia, os danos cerebrais apresentados pelos pacientes acometidos pela HMGA podem ser também uma consequência da desregulação no citoesqueleto causada por níveis tóxicos destes ácidos orgânicos, já que esses podem exercer sinergicamente um papel crítico nas vias de sinalização que regulam a fosforilação dos FI e bem como suas funções celulares.

Finalmente, é difícil de prever a importância fisiopatológica dos dados apresentados nesta investigação, pois as concentrações cerebrais de HMG, MGA e MGT ainda não foram estabelecidas no cérebro dos doentes com HMGA. No entanto, uma vez que os sintomas neurológicos aparecem ou pioram durante as crises catabólicos, em que as concentrações dos metabólitos acumulados aumentam drasticamente durante esses episódios, é

concebível que o acúmulo desses metabolitos possam estar relacionados com a piora clínica. Devemos também enfatizar que as concentrações dos ácidos orgânicos em geral, e em especial de ácidos dicarboxílicos (tais como a HMG, MGA e MGT), que se acumulam em vários acidemias orgânicas são mais elevados no cérebro, em comparação com o sangue, provavelmente devido ao baixo efluxo destes compostos a partir do cérebro, que está de acordo com a teoria de aprisionamento cerebral de ácidos dicarboxílicos (HOFFMANN *et al*., 1993; HOFFMANN *et al*., 1994; SAUER *et al*., 2006; STELLMER *et al*., 2007). E, uma vez que os resultados obtidos na presente tese podem estar diretamente envolvidos em várias doenças neurodegenerativas, somos tentados a propor que estratégias terapêuticas para regular os receptores NMDA, o estresse oxidativo / nitrosativo e os níveis de citocinas podem potencialmente servir como terapia adjuvante para a proteção do sistema nervoso central dos pacientes afetados por HMGA.

IV CONCLUSÕES

- A injeção intraestriatal dos ácidos HMG e MGA induziram a produção de espécies reativas de oxigênio e nitrogênio, a peroxidação lipídica, a oxidação protéica, diminuiram os níveis de GSH e desestabilizaram as defesas antioxidantes enzimáticas indicando que esses metabólitos induzem estresse oxidativo *ex vivo* em estriado de ratos jovens.
- A exposição de astrócitos cultivados de córtex cerebral de ratos neonatos ao HMG e ao MGA causa aumento na produção de espécies reativas, diminuição dos níveis de GSH, aumento na liberação de citocinas pró-inflamatórias mediado pela cascata da Erk e um desequilíbrio da função mitocondrial, configurando portanto, uma indução de extresse oxidativo e uma indução de resposta inflamatória nessas células neurais importantes para o funcionamento do sistema nervoso central.
- Os ácidos HMG, MGA e MGT provocaram hipofosforilação das proteínas GFAP e das subunidades NFL, NFM e NFH do citoesqueleto de estriado e de córtex cerebral de ratos jovens, mediada pela inibição das proteínas cinases PKA e JNK secundariamente ao estímulo dos receptores glutamatérgicos sinápticos e extrasinápticos bem como de dano oxidativo a essas proteínas.
- Em resumo, os principais ácidos orgânicos acumulados na HMGA provocam um desequilíbrio da homeostase redox e alteração do sistema fosforilante de proteínas do cicloesqueleto com o

envolvimento de receptores NMDA que potencialmente representam mecanismos patogênicos envolvidos no dano estriatal e cortical em pacientes afetados pela deficiência da HL.

V PERSPECTIVAS

- Estudar os efeitos da injeção intraestriatal dos ácidos HMG e MGA em ratos jovens sobre a fosforilação das proteínas do citoesqueleto no estriado.
- Estudar os efeitos dos ácidos HMG e MGA sobre a fosforilação das proteínas do citoesqueleto em culturas de astrócitos e neurônios de córtex cerebral e estriado de ratos.
- Fazer análise histopatológica com hematoxilina/eosina e imunohistoquímica para GFAP e NeuN em estriado de ratos jovens injetados com os ácidos HMG e MGA.
- Estudar a morfologia e a viabilidade celular de neurônios e astrócitos cultivados de córtex cerebral e estriado de ratos expostos aos ácidos HMG e MGA.

Referencias bibliográficas

Ackerley S, Grierson AJ, Banner S, Perkinton MS, Brownless J, Byers HL, Ward M, Thornhill P, Hussian K, Waby JS, Anderton BH, Cooper JD, Dingwall C, Leigh PN, Shaw CE, Miller CC, 2000. p38alpha Stress-activated protein kinase phosphorylates neurofilaments and is associated with neurofilament pathology in amyotrophic lateral sclerosis. Mol Cell Neurosci 26:354-64.

Akashi K, Kakizaki T, Kamiya H, Fukaya M, Yamasaki M, Abe M, Natsume R, Watanabe M, Sakimura K, 2009. NMDA receptor GluN2B (GluR epsilon 2/NR2B) subunit is crucial for channel function, postsynaptic macromolecular organization, and actin cytoskeleton at hippocampal CA3 synapses. J Neurosci 29:10869-82.

Alarcón-Aguilar A, González-Puertos VY, Luna-Lopéz A, López-Macay A, Morán J, Santamaría A, Königsberg M, 2014. Comparing the effects of two neurotoxins in cortical astrocytes obtained from rats of different ages: involvment of oxidative damage. J Appl Toxicol 34:127-38.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter PO, 2008. Citoesquleto. In: Biologia molecular da célula. 6th ed. pp. 907-82.

Al-Chalabi A e Miller CC, 2003. Neurofilaments and neurological disease. Bioessays 25:346-55.

Al-Sayed M, Imtiaz F, Alsmadi OA, Rashed MS, Meyer BF, 2006. Mutations underlying 3-hydroxy-3-methylglutaril CoA lyase deficiency in the Saudi population. BMC Med Genet 16:7-86.

Anderson LJ, Wyatt KM, Henley W, Nikolaou V, Waldek S, Hughes DA, Pastores GM, Logan S, 2014a. Long-term effectiveness of enzyme replacement therapy in Fabry disease: results from the NCS-LSD cohort study. J Inherit Metab Dis 37:969-78.

Anderson LJ, Henley W, Wyatt KM, Nikolaou V, Hughes DA, Waldek S, logan S, 2014b. Long-term effectiveness of enzyme replacement thereapy in adults

with Gaucher disease: results from the NCS-LSD cohort study. J Inherit Metab Dis 37:953-60.

Anisimov VN, 2006. Premature ageing prevention: limitations and perspectives of pharmacological interventions. Curr Drug Targets 7:1485-1503.

Ayata C, Ayata G, Hara H, Matthews RT, Beal MF, Ferrante RJ, Endres M, Kim A, Christie RH, Waeber C, Huang PL, Hyman BT, Moskowitz MA, 1997. Mechanisms of reduced striatal NMDA excitotoxicity in type I nitric oxide synthase knock-out mice. J Neurosci 17:6908-17.

Bast A, 1993. Oxidative stress and calcium homeostasis. In: Halliwell B, Aruoma OI, eds. DNA and free radicals. Chichester: Ellis Horwood, pp. 95-108.

Bear JE, Svitkina TM, Krause M, Schafer DA, Loureiro JJ, Strasser GA, Maly IV, Chaga OY, Cooper JA, Borisy GG, Gertler FB, 2002. Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblasts motility. Cell 109:509-21.

Beaulieu JM, Robertson J, Julien JP, 1999. Interactions between peripherin and neurofilaments in cultured cells: disruption of peripherin assembly by the NF-M and NF-H subunits. Biochem Cell Biol 77:41-5.

Bergendi L, Benes L, Durackova Z, Ferencik M, 1999. Chemistry, physiology and pathology of free radicals. Life Sci 65:1865-74.

Bobermin LD, Quincozes-Santos A, Guerra MC, Leite MC, Souza DO, Gonçalves CA, Gottfried C, 2012. Resveratrol prevents ammonia toxicity in astroglial cells. PLoS One 7:e52164.

Bonafé L, Troxler H, Kuster T, Heizmann CW, Chamoles NA, Burlina AB, Blau N, 2000. Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic acidurias. Mol Gen Metab 69:302-11.

Boveris A e Chance B, 1973. The mitocondrial generation of hydrogen peroxide. Biochem J 134:707-16.

Brenner M, 2014. Role of GFAP in CNS injuries. Neurosci Lett 565:7-13.

Brown GC, 2010. Nitric oxide and neuronal death. Nitric Oxide 23:153-65.

Burton BK, 1987. Inborn Errors of Metabolism: The clinical diagnosis in early infancy. Pediatrics 79:359-69.

Carmignoto G, 2000. Reciprocal communication system between astrocytes and neurones. Prog Neurobiol 62:561-81.

Carraway CAC, 2000. The cytoskeleton in transduction of signal and regulation of cellular function. In: Carraway KL e Carraway CAC, eds. Cytoskeleton: signaling and cellular regulation. New York, Ocford University Press, pp. 1-7.

Castoldi AF, Coccini T, Manzo L, 2001. Biological markers of neurotoxic diseases. Funct Neurol 16:39-44.

Chae HZ, Kang SW, Rhee SG, 1999. Isoforms of mammalian peroxiredoxin that reduce peroxides in presence of thioredoxin. Methods Enzymol 300:219-26.

Chalmers RA, Purkiss P, Watts RW, Lawson AM, 1980. Screening for organic acidurias and amino acidopathies in newborn and children. J Inherit Metab Dis 3:27-43.

Chalmers RA e Lawson LA, 1982. Organic acids in man: the analytical, chemistry, biochemistry and diagnosis of organic acidurias. London: Chapman and Hall.

Chance B, Sies H, Boveris A, 1979. Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527-605.

Chang L e Goldman RD, 2004. Intermediate filaments mediate cytoskeletal crosstalk. Nat Rev Mol Cell Biol 5:601-13.

Choi JH, Cho YM, Suh KS, Yoon HR, Kim GH, Kim SS, Ko JM, Lee JH, Park YS, Yoo HW, 2008. Short-term efficacy of enzyme replacement therapy in korean patients with Fabry disease. J Korean Med Sci 23, 243-250.

Chou YH, Skalli O, Goldman RD, 1997. Intermediate filaments and cytoplasmic networking: new connections and more functions. Curr Opin Cell Biol 9:49-53.

Circu ML e Aw TY, 2010. Reactive oxygen species, cellular redox systems, and apoptosis, Free Radic Biol Med 48:749-62.

Corcoran KA, Leaderbrand K, Radulovic J, 2013. Extinction of remotely acquired fear depends on an inhibitory NR2B/PKA pathway in the retrosplenial cortex. J Neurosci 33:19492-8.

Coulombe PA, Bousquet O, Ma L, Yamada S, Wirtz D, 2000. The 'ins' and 'outs' of intermediate filament organization. Trends Cell Biol 10:420-8.

da Rosa MS, Seminotti B, Amaral AU, Fernandes CG, Gasparotto J, Moreira JC, Gelain DP, Wajner M, Leipnitz G, 2013. Redox homeostasis is compromised in vivo by the metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency in rat cerebral cortex and liver. Free Radic Res 47:1066-75.

Darlington CL, 2005. Astrocytes as targets for neuroprotective drugs. Curr Opin Investig Drugs 6:700-3.

Dasouki M, Buchanan D, Mercer N, Gibson KM, Thoene J, 1987. 3-Hydroxy-3-methylglutaric aciduria: response to carnitine therapy and fat and leucine restriction. J Inherit Metab Dis 10:142-6.

Delanty N e Dichter MA, 1998. Oxidative injury in the nervous system. Acta Neurol Scand 98:145-53.

DePianto D e Coulombe PA, 2004. Intermediate filaments and tissue repair. Exp Cell Res 301:68-76.

DiProspero NA, Chen EY, Charles V, Plomann M, Kordower JH, Tagle DA, 2004. Early changes in Huntington's disease patient brains involve alterations in cytoskeletal and synaptic elements. J Neurocytol 33:517-33.

Draper HH e Hadley M, 1990. A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde. Xenobiotica 20:901-7.

Dringen R, 2000. Metabolism and functions of glutathione in brain. Prog Neurobiol 62:649-71.

Duprat F, Guillemare E, Romey G, Fink M, Lesage F, Lazdunski M, Honore E, 1995. Susceptibility of cloned K^+ channels to reactive oxygen species. Proc Natl Acad Sci U S A 92:11796-800.

Eckes B, Dogic D, Colucci-Guyon E, Wang N, Maniotis A, Ingber D, Merckling A, Langa F, Aumailley M, Delouvée A, Koteliansky V, Babinet C, Krieg T, 1998. Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts. J Cell Sci 111:1897-907.

Eddleston M e Mucke L, 1993. Molecular profile of reactive astrocytes-implications for their role in neurologic disease. Neuroscience 54:15-36.

Eliasson C, Sahlgren C, Berthold CH, Stakeberg J, Celis JE, Betsholtz C, Eriksson JE, Pekny M, 1999. Intermediate filament protein partnership in astrocytes. J Biol Chem 274:23996-24006.

Eng LF, Lee YL, Kwan H, Brenner M, Messing A, 1998. Astrocytes cultured from transgenic mice carrying the added human glial fibrillary acidic protein gene contain Rosenthal fibers. J Neurosci Res 53:353-60.

Eriksson JE e Goldman RD, 1993. Protein phosphatase inhibitors alter cytoskeletal structure and cellular morphology. Adv Prot Phosphatases 7:335-57.

Eriksson JE, He T, Trejo-Skalli AV, Härmälä-Braskén AS, Hellman J, Chou YH, Goldman RD, 2004. Specific in vivo phosphorylation sites determine the assembly dynamics of vimentin intermediate filaments. J Cell Sci 29:919-32.

Eriksson JE, Dechat T, Grin B, Helfand B, Mendez M, Pallari HM, Goldman RD, 2009. Introducing intermediate filaments: from discovery to disease. J Clin Invest 119:1763-71.

Erta M, Quintana A, Hidalgo J, 2012. Interleukin-6, a major cytokine in the central nervous system. Int J Biol Sci 8:1254-66.

Esterbauer H, Schaur RJ, Zollner H, 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11:81-128.

Farina C, Aloisi F, Meinl E, 2007. Astrocytes are active players in cerebral innate immunity. Trends Immunol 28:138-45.

Faull KF, Bolton PD, Halpern B, Hammond J, Danks DM, 1976a. The urinary organic acid profile associated with 3-hydroxy-3-methylglutaric aciduria. Clin Chim Acta 73:553-9.

Faull K, Bolton PD, Halpern B, Hammond J, Danks D, Hahnel R, Wilkinson SP, Wysocki SJ, Masters PL, 1976b. Letter: Patient with defect in leucine metabolism. N Engl J Med 294:1013.

Ferreira ALA e Matsubara LS, 1997. Radicais livres: conceitos, doenças relacionadas, sistema de defesa e estresse oxidativo. Rev Ass Med Brasil 43:61-8.

Fuchs E, 1994. Intermediate filaments and disease: mutations that cripple cell stregth. J Cell Biol 125:511-6.

Fuchs E e Cleveland DW, 1998. A structural scaffolding of intermediate filaments in health and disease. Science 279:514-9.

Funchal C, Zamoner A, dos Santos AQ, Loureiro SO, Wajner M, Pessoa-Pureur R, 2005a. Alpha-ketoisocaproic acid increases phosphorylation of intermediate filament proteins from rat cerebral cortex by mechanisms involving Ca^{2+} and cAMP. Neurochem Res 30:1139-46.

Funchal C, Zamoner A, dos Santos AQ, Moretto MB, Rocha JB, Wajner M, Pessoa-Pureur R, 2005b. Evidence that intracellular Ca^{2+} mediates the effect of alpha-ketoisocaproic acid on the phosphorylating system of cytoskeletal proteins from cerebral cortex of immature rats. J Neurol Sci 238:75–82.

Funghini S, Pasquini E, Cappellini M, Donati MA, Morrone A, Fonda C, Zammarchi E, 2001. 3-Hydroxy-3-methylglutaric aciduria in an Italian patient is caused by a new nonsense mutation in the HMGDL gene. Mol Genet Metab, 73:268-75.

Geisler N e Weber K, 1981. Self-assembly in vitro of the 68,000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate-sized filaments. J Mol Biol 151:565-71.

Geisler N, Vandekerckhove J, Weber K, 1987. Location and sequence characterization of the major phosphorylation sites of the high molecular mass neurofilament proteins M and H. FEBS Lett 221:403-7.

Giasson BI e Mushynski WE, 1997. Study of proline-directed protein kinases involved in phosphorylation of the heavy neurofilament subunit. J Neurosci 17:9466-72.

Gibson KM, Cassidy SB, Seaver LH, Wanders RJ, Mitchell GA, Spark RP, 1994. Fatal cardiomyopathy associated with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. J Inherit Metab Dis 17:291-4.

Gloire G, Legrand-Poels S, Piette J, 2006. NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 72:1493-1505.

Godsel LM, Hobbs RP, Green KJ, 2007. Intermediate filament assembly: dynamics to disease. Trends Cell Biol 18:28-37.

Goedert M, 1998. Filamentous nerve cell inclusions in neurodegenerative diseases. Curr Opin Neurobiol 8:619-32.

Gohara R, tang D, Inada H, Inagaki M, Takasaki Y, Ando S, 2001. Phosphorylation of vimentin head domain inhibits interaction with the carboxyl-terminal end of alpha-helical rod domain studied by surface plasmon resonance measurements. FEBS lett 489:182-6.

Gonda Y, Nishizawa K, Ando S, Kitamura S, minoura Y, Nishi Y, Inagaki M, 1990. Involvement of protein kinase C in the regulation of assembly-

disassembly of neurofilaments in vitro. Biochem Biophys res Commun 167:1316-25.

Gonzales M, Weskler B, Tsuruta D, Goldman RD, Yoon KJ, Hopkinson SB, Flitney FW, Jones JC, 2001. Structure and function of a vimentin-associated matrix adhesion in endothelial cells. Mol Biol Cell 12:85-100.

Gotow T, Takeda M, Tanaka T, Hashimoto PH, 1992. Macromolecular structure of reassembled neurofilaments as revealed by the quick-freeze deep-etch mica method: difference between NF-M and NF-H subunits in their ability to form cross-bridges. Eur J Cell Biol 58:331-45.

Gotow T, Tanaka T, Nakamura Y, Takeda M, 1994. Dephosphorylation of the largest neurofilament subunit protein influences the structure of crossbridges in reassemblied neurofilaments. J Cell Sci 107:1949-57.

Gotow T e Tanaka J, 1994. Phosphorylation of neurofilament H subunit as related to arrangement of neurofilaments. J Neurosci Res 37:691-713.

Grabowski GA, 2008. Treatment perspectives for the lysosomal storage diseases. Expert Opin Emerg Drugs 13, 197-211.

Grant P e Pant HC, 2000. Neurofilament protein synthesis and phosphorylation. J Neurocytol 29:843-72.

Gu M, Gash MT, Mann VM, Javoy-Agid F, Cooper JM, Schapira AH, 1996. Mitochondrial defect in Huntington's disease caudate nucleus. Ann Neurol 39:385-9.

Guo-Ross SX, Yang EY, Walsh TJ, Bondy SC, 1999. Decrease of glial fibrillary acidic protein in rat frontal cortex following aluminum treatment. J Neurochem 73:1609-14.

Halliwell B e Gutteridge JMC, 1996. Oxygen radicals and nervous system. Trends Neurosci 8:22-6.

Halliwell B e Gutteridge JMC, 2007. Free radicals in biology and medicine. Oxford University Press, Oxford, pp. 268-340.

Halliwell B, 2001. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 18:685-716.

Hallpike JF, Adams CWM, Tourtellotte WW, 1983. Multiple Sclerosis: pathology, diagnosis, and management. Hardcover, Williams & Wilkins.

Hardingham GE, Fukunaga Y, Bading H, 2002. Extrasynaptic NMDARs oppose synaptic NMDArs by triggering BREB shut-off and cell death pathways. Nat Neurosci 5:405-14.

Hashimoto R, Nakamura Y, Komai S, Kashiwagi Y, Tamura K, Goto T, Aimoto S, Kaibuchi K, Shiosaka S, Takeda M, 2000. Site-specific phosphorylation of neurofilament-L is mediated by calcium / calmodulin-dependent protein kinase II in the apical dendrites during long term potentiation. J Neurochem 75:373-82.

Helfand BT, Chang L, Goldman RD, 2003. The dynamic and motile properties of intermediate filaments. Annu Rev Cell Dev Biol 19:445-67.

Helfand BT, Chou YH, Shumaker DK, Goldman RD, 2005. Intermediate filaments proteins participate in signal transduction. Trends Cell Biol 15:568-70.

Henley WE, Anderson LJ, Wyatt KM, Nikolaou V, Anderson R, Logan S, 2014. The NCS-LSD cohort study: a description of the methods and analyses used to asses the long-term effectiveness of enzyme replacement therapy and substrate reduction therapy in patients with lysossomal storage disorders. J Inherit Metab Dis 37:939-44.

Hisanaga S e Hirokawa N, 1988. Structure of peripheral domais of neurofilaments revealed by low angle rotary shadowing. J Mol Biol 202:297-305.

Hoffmann GF, Meier-Augenstein W, Stockler S, Surtees R, Rating D, Nyhan WL, 1993. Physiology and pathophysiology of organic acids in cerebrospinal fluid. J Inherit Metab Dis 16:648-69.

Hoffmann GF, Gibson KM, Trefz FK, Nyhan WL, Bremer HJ, Rating D, 1994. Neurological manifestations of organic acid disorders. Eur J Pediatr 153:S94-S100.

Hoffmann GF, Von Kries R, Klose D, Lindner M, Schulze A, Muntau AC, Röschinger W, Liebl B, Mayatepek E, Roscher AA, 2004. Frequencies of inherited organic acidurias and disorders of mitochondrial fatty acid transport and oxidation in Germany. Eur J Pediatr 163:76-80.

Homan SM, Mercurio AM, LaFlamme SE, 1998. Endothelial cells assemble two distinct alpha6beta4-containing vimentin-associated structures: roles for ligant binding and the beta4 cytoplasmic tail. J Cell Sci 111: 2717-28.

Hughes DA, Mlilligan A, Mehta A, 2007. Home therapy for lysosomal storage disorders. Br J Nurs 1384:1386-9.

Inagaki M, Gonda Y, Nishizawa K, Kitamura S, Sato C, Anda S, Tanabe K, Kikuchi K, Tsuiki S, Nishi Y, 1990. Phosphorylation sites linked to glial filament disassembly, in vitro locate in a non-alpha-helical head domain. J Biol Chem 265:4722-9.

Inagaki M, Matsouka Y, Tsujimura K, Anda S, Tokui T, Takahashi T, Inagaki N, 1996. Dynamic property of intermediate filaments: regulation by phosphorylation. BioEssays 18:481-7.

Itier JM, Ret G, Viale S, Sweet L, Bangari D, Caron A, Le-Gall F, Bénichou B, Leonard J, Deleuze JF, Orsini C, 2014. Effective clearance of GL-3 human iPSC-derived cardiomyocyte model of Fabry disease. J Inherit Metab Dis 37:1013-22.

Ivaska J, Vuoriluoto K, Huovinen T, Izawa I, Inagaki M, Parker PJ, 2005. PKCepsilon-mediated phsphorylation of vimentin controls integrin recycling and motility. EMBO J 24:3834-45.

Janetzky B, Hauck S, Youdim MB, Riederer P, Jellinger K, Pantucek F, Zöchling R, Boissl KW, Reichmann H, 1994. Unaltered aconitase activity, but

decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. Neurosci Lett 169:126-128.

Janosch P, Kieser A, Eulitz M, Lovric J, Sauer G, Reichert M, Gounari F, Buscher D, Baccarini M, Mischak H, Kolch W, 2000. The Raf-1 kinase associates with vimentin kinases and regulates the structure of vimentin filaments. FASEB J 14:2008-21.

Jenner P e Olanow CW, 1996. Oxidative stress and the pathogenesis of Parkinson’s disease. Neurology 47:161-170.

Jenner P, 2003. Oxidative stress in Parkinson’s disease. Ann Neurol 53:26-38

Julien JP, 1999. Neurofilament functions in health and disease. Curr Opin Neurobiol 9:554-60.

Jung C, Yabe JT, Lee S, Shea TB, 2000. Hypophosphorylated neurofilament subunits undergo axonal transport more rapidly than more extensively phosphorylated subunits in situ. Cell Motil Cytoskeleton 47:120-9.

Kahler SG, Sherwood WG, Woof D, Lawless ST, Zaritsky A, Bonham J, Taylor CJ, Clarke JT, Durie P, Leonard JV, 1994. Pencreatitis in patients with organic acidemias. J Pediatr 124:239-43.

Kaneko S, Satoh Y, Ikemura K, Konishi T, Ohji T, Karasaki Y, Higashi K, Gotoh S, 1995. Alterations of expression of the cytoskeleton after immortalization of human fibroblasts. Cell Struct Funct 20:107-15.

Kanwar SS e Nehru B, 2007. Modulatory effects of N-acetylcysteine on cerebral cortex and cerebellum regions of ageing rat brain. Nutr Hosp 22:95-100.

Karlhuber GM, Bauer HC, Eckl PM, 1997. Cytotoxic and genotoxic effects of 4-hydroxynonenal in cerebral endothelial cells. Mutat Res 381:209-16.

Kawajiri A, Yasui Y, Goto H, Tatsuka M, Takahashi M, Nagata K, Inagaki M, 2003. Functional significance of the specific sites phosphorymated in desmin at

cleavage furrow: Aurora-B may phosphorylate and regulate type III intermediate filaments during cytokinesis coordinatedly with Rho-kinase. Mol Biol Cell 14:1489-500.

Keller JN, Hanni KB, Markerbery, WR, 1999. 4-Hydroxynonenal increases neuronal susceptibility to oxidative stress. J Neurosci Res 58:823-30

Kim S e Coulombe PA, 2007. Intermediate filament scaffolds fulfill mechanical, organizational, and signaling functions in the cytoplasm. Genes Dev 21:1581-97.

Kirkpatrick LL e Brady ST, 1999. Cytoskeleton of neurons and glia. In: Siegel G, Agranoff BW, Alberts RW, Fischer SK, Ulher MD, eds. Basic Neurochemistry - Molecular, cellular and medical aspects. 6th ed. New York, Lippincott – Raven Publishers, pp. 155-73.

Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF, 2000a. Maturation-dependent neurotoxicity of 3-hydroxyglutaric and glutaric acids in vitro: a new pathophysiologic approach to glutaryl-CoA dehydrogenase deficiency. Pediatr res 47:495-503.

Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF, 2000b. Cerebral organic acids disorders induce damage via excitotoxic organic acids in vitro. Amino Acids 18:31-40.

Korolainen MA, Auriola S, Nyman TA, Alafuzoff I, Pirttila T, 2005. Proteomic analysis of glial fibrillary acidic protein in Alzheimer's disease and aging brain. Neurobiol Dis 20:858-70.

Kosako H, Amano M, Yanagida M, Tanabe K, Nishi Y, Kaibuchi K, Inagaki M, 1997. Phosphorylation of glial fbrillary acidic protein at the same sites by cleavage furrow kinase and Rho-associated kinase. J Biol Chem 272:10333-6.

Kreis S, Schonfeld HJ, Melchior C, Steiner B, Kieffer N, 2005. The intermediate filament protein vimentin binds specifically to a recombinant integrin

alpha2/beta1 cytoplasmic tail complex and co-localizes with native alpha2/beta1 in endothelial cell focal adhesions. Exp Cell Res 305:110-21.

Kroemer G e Reed JC, 2000. Mitochondrial control of cell death. Nat Med 6:513-9.

Kuchma MH, Kim JH, Muller MT, Arlen PA, 2012. Prostate cancer cell surface-associated keratin 8 and its implications for enhanced plasmin activity. Protein J 31:195-205.

Kumar N, Robidoux J, Daniel KW, Guzman G, Floering LM, Collins S, 2007. Requirement of vimentin filament assembly for beta3-adrenergic receptor activation of ERK MAP kinase and lipolysis. J Biol Chem 282:9244-50.

Larivieri RC e Julien JP, 2004. Functions of intermediate filaments in neuronal development and disease. J Neurobiol 58:131-48.

Lee VM, Otvos LJr, Carden MJ, Hollosi M, Dietzschold B, Lazzarini RA, 1988. Identification of the major multiphosphorylation site in mammalian neurofilaments. Proc Natl Acad Sci USA 85:1998-2002.

Lee M, Cho T, Jantaratnotai N, Wang YT, McGeer E, McGeer PL, 2010. Depletion of GSH in glial cells induces neurotoxicity: relevance to aging and degenerative neurological diseases. FASEB J 24:2533-45.

Leipnitz G, Seminotti B, Haubrich J, Dalcin KB, Solano A, de Bortoli G, Rosa RB, Amaral AU, Dutra-Filho CS, Latini A, Wajner M, 2008a. Evidence that 3-hydroxy-3-methylglutaric acid promotes lipid and protein oxidative damage and reduces the nonenzymatic antioxidant defenses in rat cerebral cortex. J Neurosci Res 86:683-93.

Leipnitz G, Seminotti B, Amaral AU, de Bortoli G, Solano A, Schuck PF, Wyse AT, Wannmacher CM, Latini A, Wajner M, 2008b. Induction of oxidative stress by the metabolites accumulating in 3-methylglutaconic aciduria in cerebral cortex of young rats. Life Sci 82:652-62.

Leipnitz G, Seminotti B, Fernandes CG, Amaral AU, Beskow AP, da Silva Lde B, Zanatta A, Ribeiro CA, Vargas CR, Wajner M, 2009. Striatum is more vulnerable to oxidative damage induced by the metabolites accumulating in 3-hydroxy-4-methylglutaryl-CoA lyase deficiency as compared to liver. Int J Dev Neurosci 27:351-6.

Leung AAC, Chan AK, Ezekowitz JA, Leung AKC, 2009. A case of dilated cardiomyopathy associated with 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) lyase deficiency. Case Rep Med 2009:183125.

Li BS, Veeranna KT, Gu J, Grant P, Pant HC, 1999a. Activation of mitogen-activated protein kinases (Erk1 and Erk2) cascade results in phosphorylation of NF-M tail domains in transfected NIH 3T3 cells. Eur J Biochem 262:211-7.

Li BS, Veeranna KT, Gu J, Grant P, Pant HC, 1999b. Calcium influx and membrane depolarization induce phosphorylation of neurofilament (NF-M) KSPrepeats in PC12 cells. Brain Res Mol Brain Res 70:84-91.

Liu D, Wen J, Liu J, Li L, 1999. The roles of free radicals in amyotrophic lateral sclerosis: reactive oxygen species and elevated oxidation of protein, DNA, and membrane phospholipids. FASEB J 13:2318-28.

Liu Q, Xie F, Siedlak SL, Nunomura A, Honda K, Moreira PI, Zhua X, Smith MA, Perry G, 2004. Neurofilament proteins in neurodegenerative diseases. Cell Mol Life Sci 61:3057-75.

Lönnrot K, Metsä-Ketelä T, Molnár G, Ahonen JP, Latvala M, Peltola J, Pietilä T, Alho H, 1996. The efect of ascorbic acid and ubiquinone supplementation on plasma and CSF total antioxidant capacity. Free Radic Biol Med 21:211-7.

Loureiro SO, Heimfarth L, Lacerda BA, Vidal LF, Soska A, dos Santos NG, de Souza Wyse AT, Pessoa-Pureur R, 2010a. Homocysteine induces hypophosphorylation of intermediate filaments and reorganization of actin cytoskeleton in C6 glioma cells. Cell Mol Neurobiol 30:557-68.

Loureiro SO, Romão L, Alves T, Fonseca A, Heimfarth L, Moura Neto V, Wyse AT, Pessoa-Pureur R, 2010b. Homocysteine induces cytoskeletal remodeling and production of reactive oxygen species in cultured cortical astrocytes. Brain Res 1355:151-64.

Lovell MA, Xie C, Markesbery WR, 2000. Decreased base excision repair and increased helicase activity in Alzheimer's disease brain. Brain Res 855:116-23

Manning G, plowman GD, Hunter T, Sudarsanam S, 2002a. Evolution of protein kinase signaling from yeast to man. Trends Biochem Sci 27:514-20.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S, 2002b. The protein kinase complement of the human genome. Science 298:1912-34.

Mark RJ, Lovell MA, Markesbery WR, Uchida K, Mattson MP, 1997. A role for 4-hydroxynonenal, an aldehydicproduct of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid beta-peptide. J Neurochem 68:255-64.

Markesbery WR, Carney JM, 1999. Oxidative alterations in Alzheimer's disease. Brain Pathol 9:133-46.

Maxwell SR, 1995. Prospects for the use of antioxidant therapies. Drugs 49:345-61.

Mayatepek E, Hoffmann GF, Baumgartner R, Schulze A, Jakobs C, Trefz FK, Bremer HJ, 1996. Atypical vitamin B12-unresponsive methylmalonic aciduria in sibship with severe progressive encephalomyelopathy: a new genetic disease? Eur J Pediatr 155:398-403.

McCall MA, Gregg RG, Behringer RR, Brenner M, Delaney CL, Galbreath EJ, Zhang CL, Pearce RA, Chiu SY, Messing A, 1996. Targeted deletion in astrocyte intermediate filament (GFAP) alters neuronal physiology. Proc Natl Acad Sci USA 93:6361-6.

McConnell EJ, Bittelmeyer AM, Raess BU, 1999. Irreversible inhibition of plasma membrane ($Ca^{2+}Mg^{2+}$)-ATPase and Ca^{2+} transport by 4-OH-2,3-trans-nonenal. Arch Biochem Biophys 361:252-6.

Menao S, López-Viñas E, Mir C, Puisac B, Gratacós E, Arnedo M, Carrasco P, Moreno S, Ramos M, Gil MC, Pie A, Ribes A, Pérez-Cerda C, Ugarte M, Clayton PT, Korman SH, Serra D, Asins G, Ramos FJ, Gómez-Puertas P, Hegardt FG, Casals N, Pié J, 2009. Tem novel HMGCL mutations in 24 patients of different origin with 3-hydroxy-3-methylglutaric aciduria. Hum Mutat, 30:520-9.

Menet V, Gimenez y Ribotta M, Chauvet N, Drian MJ, Lannoy J, Colucci-Guyon E, Privat A, 2001. Inactivation of the glial fibrillary acidic protein gene, but not that of vimentin, improves neuronal survival an neurite growth by modifying adhesion molecule expression. J Neurosci 21:6147-58.

Middeldorp J e Hol EM, 2011. GFAP in health and disease. Prog Neurobiol 93:421-43.

Miller CC, Ackerley S, Brownless J, Grierson AJ, Jacobsen NJ, Thornhill P, 2002. Axonal transport of neurofilaments in normal and disease states. Cell Mol Life Sci 59:323-30.

Mitchell GA e Fukao TJC, 2001. Inborn errors of ketone body metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill pp. 2340-2342.

Mota SI, Ferreira IL, Pereira C, Oliveira CR, Rego AC, 2012. Amyloid-beta peptide 1-42 causes microtubule deregulation through N-methyl-D-aspartate receptors in mature hippocampal cultures. Curr Alzheimer Res 9:844-56.

Motil J, Chan WK, Dubey M, Chaudhury P, Pimenta A, Chylinski TM, Ortiz DT, Shea TB, 2006. Dynein mediates retrograde neurofilament transport within axons and anterograde delivery of NFs from perikaria into axons: regulation by multiple phosphorylation events. Cell Motil Cytoskeleton 63:266-86.

Nakamura Y, Takeda M, Aimoto S, Hojo H, Takao T, Shimonishi Y, Hariguchi S, Nishimura T, 1992. Assembly regulatory domain of glial fibrillary acidic protein. A single phosphorylation diminishes its assembly-accelerating property. J Biol Chem 267:23269-74.

Ndozangue-Touriguine O, Hamelin J, Breard J, 2008. Cytoskeleton and apoptosis. Biochem Pharmacol 76:11-8.

Nestler EJ e Greengard P, 1999. Serine and threonine phosphorylation. In: Siegel G, Agranoff BW, Alberts RW, Fisher SK, Ulher MD, eds. Basic Neurochemistry – molecular, cellular and medical aspect. 6th ed. New York, Lippincott, Raven Publishers, pp. 472-95.

Nieminem M, Henttinen T, Merinen M, Marttila-Ichihara F, Erikson JE, Jalkanen S, 2006. Vimentin function in lymphocyte adhesion and transcellular migration. Nat Cell Biol 8:156-62.

Nixon RA e Shea TB, 1992. Dynamics of neuronal intermediate filaments: a developmental perspective. Cell Motil Cytoskeleton 22:81-91.

Nizuma K, Endo H, Chan PH, 2009. Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. J Neurochem 109:133-8.

Nourooz-Zadeh J, Liu EH, Yhlen B, Anggard EE, Halliwell B, 1999. F4-isoprostanes as specific marker of docosahexaenoic acid peroxidation in Alzheimer's disease. J Neurochem 72:734-40.

Omary MB e Ku NO, 1997. Intermediate filament proteins of the liver: emerging disease association and functions. Hepatology 25:1043-8.

Omary MB, Coulombe PA, McLean WH, 2004. Intermediate filament proteins and their associated disease. N Engl J Med 351:2087-2100.

Omary MB, Ku NO, Tao GZ, Toivola DM, Liao J, 2006. "Heads and tails" of intermediate filament phosphorylation: multiple sites and functional insights. Trends Biochem Sci 31:383-94.

Ong WY, Lu XR, Hu CY, Halliwell B, 2000. Distribution of hydroxynonenal-modified proteins in the kainate-lesioned rat hippocampus: evidence that hydroxynonenal formation precedes neuronal cell death. Free Radic Biol Med 28:1214-21.

Ortiz A, Oliveira JP, Wanner C, Brenner BM, Waldek S, Warnock DG, 2008. Recommendations and guidelines for the diagnosis and treatment of Fabry nephropathy in adults. Nat Clin Pract Nephrol 4, 327-36.

Pallari HM e Eriksson JE, 2006. Intermediate filaments as signaling platforms. Sci STKE 2006:pe53.

Paramio JM e Jorcano JL, 2002. Beyond structure: do intermediate filaments modulate cell signaling? Bioessays 24:836-44.

Pekny M, Johansson CB, Eliasson C, Stakeberg J, Wallen A, Perlmann T, Lendahl U, Betsholtz C, Berthold CH, Frisen J, 1999. Abnormal reaction to central nervous system injury in mice lacking glial fibrillary acidic protein and vimentin. J Cell Biol 145:503-14.

Perlson E, Hanz S, Ben-Yaakov K, Segal-Ruder Y, Seger R, Fainzilber M, 2005. Vimentin-dependent spatial translocation of na activated MAP kinase in injured nerve. Neuron 45:715-26.

Perry G, Taddeo MA, Petersen RB, Castellani RJ, Harris PL, Siedlak SL, Cash AD, Liu Q, Nunomura A, Atwood CS, Smith MA, 2003. Adventiously-bound redox active iron and copper are at the center of oxidative damage in Alzheimer disease. Biometals 16:77-81.

Petzold A, 2005. Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. J Neurol Sci 233:183-98.

Pié J, Casals N, Puisac B, Hegardt FG, 2003. Molecular basis of 3-hydroxy-3-methylglutaric aciduria. J Physiol Biochem 59:311-21.

Pié J, López-Viñas E, Puisac B, Menao S, Pie A, Casale C, Ramos FJ, Hegardt FG, Gómez-Puertas P, Casals N, 2007. Molecular genetics of HMG-CoA lyase deficiency. J Inherit Metab 92:198-229.

Pierozan P, Zamoner A, Soska ÂK, de Lima BO, Reis KP, Zamboni F, Wajner M, Pessoa-Pureur R, 2012. Signaling mechanisms downstream of quinolinic acid targeting the cytoskeleton of rat striatal neurons and astrocytes. Exp Neurol 233:391-9.

Pierozan P, Gonçalves Fernandes C, Ferreira F, Pessoa-Pureur R, 2014. Acute intrastriatal injection of quinolinic acid provokes long-lasting misregulation of the cytoskeleton in the striatum, cerebral cortex and hippocampus of young rats. Brain Res 1577:1-10.

Pope SA, Milton R, Heales SJ, 2008. Astrocytes protect against copper-catalysed loss of extracellular glutathione. Neurochem Res 33:1410-8.

Pospísilová E, Mrázová L, Hrdá J, Martincová O, Zeman J, 2003. Biochemical and molecular analyses in three patients with 3-hydroxy-3-methylglutaric aciduria. J inherit Metab Dis 26:433-41.

Purves D, Augustine GJ, Fitzpatrick D, Katz LC, Lamantia AS, McNamara JO, Williams SM, 2005. Transdução intracelular de sinal. In: Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia AS, McNamara JO, eds. Neurociências. 2nd ed. Porto Alegre, Artmed, pp. 165-85.

Ralton JE, Lu X, Hutcheson AM, Quilan RA, 1994. Identification of two N-terminal non-alpha-helical domain motifs important in the assembly of glial fibrillary acidic protein. J Cell Sci 107:1935-48.

Rashed M, Ozand PT, al Aqeel A, Gascon GG, 1994. Experience of King Faisal Specialist Hospital and Research Center with Saudi organic acid disorders. Brain Dev 16:1-6.

Reimão S, Morgado C, Almeida IT, Silva M, Corte Real H, Campos J, 2009. 3-Hydroxy-3-methylglutaryl-coenzyme A lyase deficiency: initial presentation in a young adult. J Inherit Metab Dis 32:S49-S52.

Reiter RJ, Tan DX, Manchester LC, Qi W, 2001. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. Cell Biochem Biophys 34:237-56.

Ribeiro CA, Hickmann FH, Wajner M, 2011. Neurochemical evidence that 3-methylglutaric acid inhibits synaptic Na+,K+-ATPase activity probably through oxidative damage in brain cortex of young rats. Int J Dev Neurosci 29:1-7.

Sanchez C, Diaz-Nido J, Avila J, 2000. Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. Prog Neurobiol 61:133-68.

Santello M, Bezzi P, Volterra A, 2011. TNFα controls glutamatergic gliotransmission in the hippocampal dentate gyrus. Neuron 69:988-1001.

Sarafian TA, Bredesen DE, Verity MA, 1996. Cellular resistence to methylmercury. Neurotoxicology 17:27-36.

Sauer SW, Okun JG, Fricker G, Mahringer A, Müller I, Crnic LR, Mühlhausen C, Hoffmann GF, Hörster F, Goodman SI, Harding CO, Koeller DM, Kölker S, 2006. Intracerebral accumulation of glutaric and 3-hydroxyglutaric acids secondary to limited flux across the blood-brain barrier constitutes a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. J Neurochem 97:899-910.

Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD, 1989. Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1:1269.

Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, Marsden CD, 1990a. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 54:823-7.

Schapira AHV, Mann VM, Cooper JM, Dexter D, Daniel SE, Jenner P, Clark JB, Marsden CD, 1990b. Anatomic and disease specificity of NADH CoQ1reductase (complex I) deficiency in Parkinson's disease. J Neurochem 55:2142-5.

Schaumburg HH, Powers JM, Raine CS, Suzuki K, Richardson EPJr, 1975. Adrenoleukodystrophy. A clinical and pathological study of 17 cases. Arch Neurol 32:577-91.

Scriver CR, Beaudet AL, Sly WS, Valle D, 2001. The metabolic and molecular bases of inherited disease. 8th ed. New York: McGraw-Hill Inc, pp. 3-45.

Sekimata M, Tsujimura K, Tanaka J, Takeuchi Y, Inagaki N, Inagaki M, 1996. Detection of protein kinase activity specifically activated at metaphase-anaphase transition. J Cell Biol 132:635-41.

Sies H, 1985. Oxidative stress: introductory remarks. In: SIES H, ed. Oxidative Stress; London: Academic Press.

Sihag RK e Nixon RA, 1990. Phosphorylation of the amino-terminal head domain of the middle molecular mass 145-kDa subunit of neurofilaments. Evidence for regulation by second messenger-dependent protein kinases. J Biol Chem 265:4166-71.

Sihag RK, Jaffe H, Nixon RA, Rong X, 1999. Serine-23 is a major protein kinase A phosphorylation site on the amino-terminal head domain of the middle molecular mass subunit of neurofilament proteins. J neurochem 72:491-9.

Sihag RK, Inagaki M, Yamaguchi T, Shea TB, Pant HC, 2007. Role of phosphorylation on the structural dynamics and functional of types III and IV intermediate filaments. Exp Cell Res 313:2098-109.

Simon G, Erdos M, Maródi L, Tóth J, 2008. Gaucher disease: The importance of early diagnosis and therapy. Orv Hetl 149:743-50.

Sinclair LA, 1982. A new look at the inborn errors of metabolism. Ann Clin Biochem, 19:314-21.

Singh P, Jain A, Kaur G, 2004. Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage. Mol Cell Biochem 260:153-9.

Smith CD, Carney JM, Starke-Reed PE, Oliver CN, Stadtman ER, Floyd RA, Markesbery WR, 1991. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer's disease. Proc Natl Acad Sci USA 88:10540-3.

Southorn PA e Powis G, 1988. Free radicals in medicine. I. Chemical nature and biological reactions. Mayo Clin Proc 63:381-9.

Steinert PM e Roop DR, 1988. Molecular and cellualr biology of intermediate filaments. Annu Rev Biochem 57:593-625.

Stellmer F, Keyser B, Burckhardt BC, Koepsell H, Streichert T, Glatzel M, Jabs S, Thiem J, Herdering W, Koeller DM, Goodman SI, Lukacs Z, Ullrich K, Burckhardt G, Braulke T, Mühlhausen C, 2007. 3-Hydroxyglutaric acid is transported via the sodium-dependent dicarboxylate transporter NaDC3. J Mol Med (Berl) 85:763-70.

Strack S, Westaphal RS, Colbran RJ, Ebner FF, Wadzinski BE, 1997. Protein serine/threonine phosphate 1 and 2A associate with and dephosphorylate nurofilaments. Brain Res Mol Brain Res 49:15-28.

Su W, Chen HB, Li SH, Wu DY, 2012. Correlation study of the serum levels of the glial fribrillary acidic protein and neurofilament proteins in Parkinson's disease patients. Clin Neurol Neurosurg 114:371-5.

Suh SW, Gum ET, Hamby AM, Chan PH, Swanson RA, 2007. Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. J Clin Invest 117:910-8.

Sweetman L e Williams JC, 2001. Branched chain organic acidurias. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease, 8th ed. New York, McGraw-Hill, pp. 2340-2.

Takemura M, Nishiyama H, Itohara S, 2002. Distribution of phosphorylated glial fribrillary acidic protein in the mouse central nervous system. Genes Cells 7:295-307.

Tanabe K, Matsushima-Nishiwaki R, Yamaguchi S, Iida H, Dohi S, Kozawa O, 2010. Mechanisms of tumor necrosis factor-alpha-induced interleukin-6 synthesis in glioma cells. J Neuroinflammation 7:16.

Tanabe K, Kozawa O, Iida H, 2011. Midazolam suppresses interleukin-1beta-induced interleukin-6 release from rat glial cells. J Neuroinflammation 8:68.

Thompson GN, Chalmers RA, Halliday D, 1990. The contribution of protein catabolism to metabolic decompensation in 3-hydroxy-3-methylglutaric aciduria. Eur J Pediatr 149:346-50.

Toivola DM, Tao GZ, Habtezion A, Liao J, Omary MB, 2005. Cellular integrity plus: organelle-related and protein targeting functions of intermediate filaments. Trends Cell Biol 15:608-17.

Tsai MJ e Lee EH, 1994. Differences in the disposition and toxicity of 10-methyl-4-phenylpyridinium in cultured rat and mouse astrocytes. Glia 12:329-35.

Tsujimura K, Tanaka J, Ando S, Matsuoka Y, Kusubata M, Sugiura H, Yamauchi T, Inagaki M, 1994. Identification of phosphorylation sites on glial fribrillary acidic protein for cdc2 kinase and Ca(2+)-calmodulin-dependent protein kinase II. J Biochem 116:426-34.

Tsuruta D e Jones JC, 2003. The vimentin cytoskeleton regulates focal contact size and adhesion of endothelial cells subjected to shear stress. J Cell Sci 116:4977-84.

van der Knaap MS, Bakker HD, Valk J, 1998. MR imaging and protonspectroscopy in 3-hydroxy-3-methylglutaryl coenzyme A lyase deficiency. Am J Neuroradiol 19:378-82.

van Dussen L, Akkerman EM, Hollak CEM, Nederveen AJ, Maas M, 2014. Evaluation of an imaging biomarker, Dixon quantitative chemical shift imaging, in Gaucher disease: lessons learned. J Inherit Metab Dis 37:1003-11.

Vargas CR, Sitta A, Schmitt G, Ferreira GC, Cardoso ML, Coelho D, Gibson KM, Wajner M, 2007. Incidence of 3-hydroxy-3-methylglutaryl-CoA coenzyme A lyase (HL) deficiency in Brazil, South America. J Inherit Metab Dis SR #093.

Vedder AC, Breunig F, Donker-Koopman WE, Mills K, Young E, Winchester B, Ten Berge IJ, Groener JE, Aerts JM, Wanner C, Hollak CE, 2008. Treatment of Fabry disease with different dosing regimens of agalsidase: Effects on antibody formation and GL-3. Mol Genet Metab 94:319-25.

Wagner OI, Lifshitz J, Janmey PA, Linden M, McIntosh TK, Leterrier JF, 2003. Mechanisms of mitochondria-neurofilament interactions. J Neurosci 23:9046-58.

Wakabayashi N, Slocum SL, Skoko JJ, Shin S, Kensler TW, 2010. When NRF2 talks, who's listening? Antioxid Redox Signal 13:1649-63.

Wang SP, Marth JD, Oligny LL, Vachon M, Robert MF, Ashmarina L, Mitchell GA, 1998. 3-Hydrohy-3-methylglutaryl-CoA lyase (HL): gene targeting causes prenatal lethality in HL-deficient mice. Hum Mol Genet 7:2057-62.

Ward RJ e Peters TJ, 1995. Free Radicals. In: Marshall WJ, Bangert SK, eds. Clinical Biochemistry: Metabolic and Clinical Aspects. New York, Churchill Livingstone, pp. 765-77.

Xu ZS, Liu WS, Willard MB, 1992. Identification of six phosphorylation sites in the COOH-terminal tail region of the rat neurofilament protein M. J Biol Chem 267:4467-71.

Yalçinkaya C, Dinçer A, Gündüz E, Fiçicioglu C, Koçer N, Aydin A, 1999. MRI and MRS in HMG-CoA lyase deficiency. Pediatr Neurol 20:375-80.

Yang X, Wahng J, Liu C, Grizzle WE, Yu S, Zhang S, Barnes S, Koopman WJ, Mountz JD, Kimberly RP, Zhang HG, 2005. Cleavege of p53-vimentin complex

enhances tumor necrosis factor-related apoptosis-inducing ligant-mediated apoptosis of rheumatoid arthritis synovial fibroblasts. Am J Pathol 167:705-19.

Yasui Y, Amano M, Nagata K, Inagaki N, Nakamura H, Saya H, Kaibuchi K, Inagaki M, 1998. Roles of Rho-associated kinase in cytokinesis; mutations in Rho-associated kinase phosphorylation sites impair cytokinect segregation of glial filaments. J Cell Biol 143:1249-58.

Ye L, Huang Y, Zhao L, Li Y, Sun L, Zhou Y, Qian G, Zheng JC, 2013. IL-1β and TNF-α induce neurotoxicity through glutamate production: a potential role for neuronal glutaminase. J Neurochem 125:897-908.

Yuan A, Rao MV, Sasaki T, Chen Y, Kumar A, Veeranna, Liem RK, Eyer J, Peterson AC, Julien JP, Nixon RA, 2006. Alpha-internexin is structurally and functionally associated with the neurofilament triplet proteins in the mature CNS. J Neurosci 26:10006-19.

Yýlmaz Y, Ozdemir N, Ekinci G, Bayakal T, Kocaman C, 2006. Corticospinal tract involvement in a patient with 3-HMG coenzyme A lyase deficiency. Pediatr Neurol 35:139-41.

Zafeiriou DI, Vargiami E, Mayapetek E, Augoustidou-Sawopoulou P, Mitchell GA, 2007. 3-Hydroxy-3-methylglutaryl coenzyme a lyase deficiency with reversible white matter changes after treatment. Pediatr Neurol 37:47-50.

Zaleska MM e Floyd RA, 1985. Regional lipid peroxidation in rat brain in vitro: possible role of endogenous iron. Neurochem Res 10:397-410.

Zhou X, Ding Q, Chen Z, Yun H, Wang H, 2013a. Involvement of the GluN2A and GluN2B subunits in synaptic and extrasynaptic N-methyl-D-aspartate receptor function and neuronal excitotoxicity. J Biol Chem 288:24151-9.

Zhou X, Hollern D, Liao J, Andrechek E, Wang H, 2013b. NMDA receptor-mediated excitotoxicity depends on the coactivation of synaptic and extrasynaptic receptors. Cell Death Dis 4:e560.

Zhu Q, Lindenbaum M, Levavasseur F, Jacomy H, Julien JP, 1998. Disruption of the NF-H gene increases axonal microtubule content and velocity of neurofilament transport: relief of axonopathy resulting from the toxin beta,beta'-iminodipropionitrile. J Cell Biol 143:183-93.

Página eletrônica visitada: <http://www.gceadvancelevel.com/cytoskeleton/>. Acessado em: 11 de janeiro de 2015.