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 *IN VITRO* DOS ÁCIDOS 3-HIDROXI-3-METILGLUTÁRICO, 3-METILGLUTÁRICO, 3-METILGLUTACÔNICO E 3-HIDROXIISOVALÉRICO SOBRE PARÂMETROS DE ESTRESSE OXIDATIVO EM CÉREBRO E FÍGADO DE RATOS JOVENS

Guilhian Leipnitz

ORIENTADOR: Prof. Dr. Moacir Wajner

CO-ORIENTADORA: Profa. Dra. Alexandra Susana Latini

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito parcial para a obtenção do título de Doutor em Bioquímica.

Porto Alegre, outubro de 2009.

À minha família

“Quando a gente acha que tem todas as respostas,
vem a vida e muda todas as perguntas ...”

Luis Fernando Verissimo

AGRADECIMENTOS

A Deus, que me guia pelos caminhos certos e me protege nos momentos mais difíceis. Agradeço por cada momento de minha vida e, acima de tudo, pelas pessoas que me amam.

Ao meu amor Kari. A quem eu amo mais a cada dia que passa. Minha companheira, meu equilíbrio, minha amiga e minha felicidade. A quem nunca conseguirei agradecer pela forma como me faz sentir. MUITO OBRIGADO POR TER ENTRADO NA MINHA VIDA. Te amo muito!

Aos meus pais, as pessoas mais especiais desse mundo. A quem devo tudo que tenho, tudo que sou e tudo que conquistei. Ao meu pai, a pessoa mais carinhosa e à minha mãe, a pessoa mais guerreira da face da Terra.

Aos meus queridos irmãos Biel e Gugu, meus amores e meus amigos. Dizem que família nós não escolhemos, mas esses dois, com certeza, eu escolhi.

Aos meus familiares, avós, tios, tias e primos; todos moram no meu coração.

A família da Kari: sogro, sogra, cunhados, avós, tios e primos, obrigado por me acolherem e serem minha família também.

Ao Prof. Moacir, uma pessoa muito importante na minha vida que me ajudou muito e sempre cuidou de mim, não apenas no lado profissional, mas também na vida.

A Lali, minha "madresita", minha amiga, minha co-orientadora, minha conselheira, pessoa muito especial que levarei comigo o resto da minha vida.

A Bi, minha bolsista querida que me ajudou muito, mas mais importante do que isso, uma grande amiga que sempre está do meu lado para o que der e vier.

A Carol Fernandes, grande amiga e alto astral, colega de trabalho e parceira de maluquices e risadas.

Ao Alexandre Amaral, colorado, parceiro e grande amigo.

Aos meus grandes amigos, pessoas especiais, que sei que poderei contar pelo resto da minha vida: Alexandre Solano, Carolzinha, César e família, Cuca, Déia, Genaro, Guto, Xuxa, Thiaguinho, Paulo.

Ao Rafa, um cara iluminado, por toda a amizade e companheirismo e pelas "grandes histórias", principalmente em Floripa.

Aos amigos que passaram pelo laboratório, pessoas muito especiais, Ana Paula, Denis, Doda, Lê e Lucila.

A Anezinha e a Carol Viegas, grandes amigas com quem posso contar sempre.

Ao Gustavo e a Pati, que me ajudaram muito ao longo desses anos.

Aos queridos colegas Ângela, Lisiane, Estela, Mateus, pessoas muito fáceis de gostar.

Ao pessoal dos outros laboratórios do grupo, especialmente a Ângela Sgaravatti, Bárbara, Cris Matté, Fran e Virgínia.

Ao pessoal da UFSC, especialmente do laboratório da Lali, pessoas muito queridas.

Aos professores Clóvis, Dutra e Ângela pela amizade e ajuda ao longo desses anos.

A UFRGS e ao Departamento de Bioquímica, minha segunda casa e a todos os professores e funcionários.

A todos os meus amigos, obrigado de coração!

SUMÁRIO

PARTE I

Introdução e Objetivos

RESUMO

As acidúrias 3-hidroxi-3-metilgutárica (HMGA) e 3-metilglutacônica (MGTA) são doenças que afetam o catabolismo da leucina e a cetogênese. Os pacientes afetados pela HMGA apresentam acúmulo e elevada excreção urinária dos ácidos 3-hidroxi-3-metilglutárico (HMG), 3-metilglutárico (MGA), 3-metilglutacônico (MGT) e 3-hidroxiisovalérico (OHIVA), ao passo que a MGTA é bioquimicamente caracterizada pelo acúmulo e aumento na excreção urinária de MGT, MGA e OHIVA. Os indivíduos afetados por MGTA apresentam predominantemente sintomas neurológicos. Já os pacientes acometidos pela HMGA também apresentam hepatomegalia, principalmente em situações de descompensação metabólica, quando ocorrem elevações drásticas nas concentrações dos metabólitos acumulados. Considerando que os mecanismos patogênicos responsáveis pelo dano neurológico encontrado nos pacientes portadores da HMGA e da MGTA são pouco conhecidos, inicialmente investigamos os efeitos *in vitro* dos ácidos HMG, MGA, MGT e OHIVA sobre importantes parâmetros de estresse oxidativo em córtex cerebral de ratos jovens. Verificamos que os metabólitos testados induziram peroxidação lipídica (aumento de substâncias reativas ao ácido tiobarbitúrico (TBA-RS) e da quimioluminescência) e dano oxidativo protéico (formação de carbonilas e oxidação de grupamentos sulfidrila). Além disso, a indução de peroxidação lipídica causada pelo HMG e pelo MGT foi prevenida pela adição de antioxidantes. Também foi demonstrado que o HMG, MGA, MGT e OHIVA diminuíram as defesas antioxidantes não-enzimáticas (diminuição das concentrações de glutationa reduzida (GSH), do potencial antioxidante total e da reatividade antioxidante total) e que a diminuição das concentrações de GSH causada pelo HMG e pelo MGA foi prevenida por antioxidantes. Por outro lado, nenhum dos ácidos orgânicos foi capaz de oxidar grupamentos sulfidrila de uma solução comercial de glutationa e de membranas mitocondriais isoladas de córtex cerebral.

Na segunda parte do trabalho, comparamos a vulnerabilidade do estriado cerebral e do fígado ao dano oxidativo causado pelo HMG, MGA, MGT e OHIVA. Verificamos que todos os ácidos orgânicos aumentaram a medida de TBA-RS no estriado, sendo que apenas o HMG, além de apresentar o efeito mais pronunciado, induziu peroxidação lipídica no fígado. Esses metabólitos também diminuíram as concentrações de GSH em estriado. Novamente apenas o HMG reduziu as defesas antioxidantes não-enzimáticas no fígado. Finalmente, nossos resultados demonstraram que o HMG, MGA e o MGT induziram a oxidação de grupamentos sulfidrila em estriado, ao passo que nenhum dos metabólitos alterou esse parâmetro em fígado. Os efeitos detectados no presente estudo demonstram que os ácidos orgânicos acumulados na HMGA e na MGTA induzem estresse oxidativo em córtex cerebral, estriado e fígado de ratos jovens. Também evidencia que os tecidos cerebrais apresentam maior vulnerabilidade que o fígado ao dano oxidativo provocado por esses metabólitos, o que está de acordo com a sintomatologia apresentada pelos pacientes que é predominantemente neurológica. Portanto, presumimos que o estresse oxidativo pode contribuir, ao menos em parte, para a fisiopatologia dos danos teciduais encontrados nos pacientes acometidos pela HMGA e MGTA.

ABSTRACT

3-Hydroxy-3-methylglutaric (HMGA) and 3-methylglutaconic (MGTA) acidurias are disorders that affect leucine catabolism and ketogenesis. HMGA-affected patients present tissue accumulation and high urinary excretion of 3-hydroxy-3-methylglutaric (HMG), 3-methylglutaric (MGA), 3-methylglutaconic (MGT) and 3-hydroxyisovaleric (OHIVA) acids, whereas MGTA is biochemically characterized by accumulation of MGT, MGA and OHIVA. MGTA- and HMGA-affected patients predominantly present neurologic symptoms. HMGA patients also present hepatomegaly, especially during metabolic crisis, which is characterized by a dramatic increase of the concentrations of the accumulating organic acids. Considering that the mechanisms of brain damage in HMGA and MGTA are poorly known, we first investigated the *in vitro* effects of HMG, MGA, MGT and OHIVA on important parameters of oxidative stress in cerebral cortex from young rats. We verified that the organic acids induced lipid peroxidation (thiobarbituric-acid reactive species, TBA-RS, and chemiluminescence increase) and protein oxidative damage (carbonyl formation and sulfhydryl oxidation) in cortical supernatants. The lipid peroxidation induced by HMG and MGT was prevented by the addition of free radical scavengers, suggesting that free radicals are involved in these effects. It was also demonstrated that HMG, MGA, MGT and OHIVA reduced non-enzymatic antioxidant defenses (reduced glutathione, GSH, concentrations, total radical-trapping antioxidant potential and total antioxidant reactivity) and that GSH decrease caused by HMG and MGA was prevented by free radical scavengers. On the other hand, the metabolites did not oxidize sulfhydryl groups from a commercial solution of GSH and from purified membrane protein-bound thiol groups, indicating that the decrease of GSH levels caused by the organic acids was not due to a direct oxidative effect.

Next, we evaluated the vulnerability of a central (striatum) and a peripheral (liver) tissue to the oxidative damage caused by HMG, MGA, MGT and OHIVA. It can be observed that HMG, MGA, MGT and OHIVA induced lipid peroxidation (TBA-RS increase) in striatum. Among the various acids, only HMG increased TBA-RS in the liver. Furthermore, HMG induced the highest degree of lipid peroxidation in the striatum. It was also verified that the organic acids diminished GSH concentrations. Again, HMG presented the strongest effect compared to the other metabolites, reducing GSH levels in the striatum and in the liver. Finally, we observed that HMG, MGT and MGA reduced the sulfhydryl content (protein oxidation) in the striatum, whereas the metabolites did not alter this parameter in the liver. The present data indicate the HMG, MGA, MGT and OHIVA induce oxidative stress in cerebral cortex, striatum and liver from young rats. It can be also observed that the brain is more vulnerable than liver to lipid and protein oxidative damage induced by these organic acids, which is in agreement with the fact that the patients present predominantly neurologic symptoms. Therefore, it may be presumed that oxidative stress elicited *in vitro* by HMG, MGA, MGT and OHIVA contributes, at least in part, to the pathophysiology of the brain and hepatic damage found in HMGA and MGTA.

LISTA DE ABREVIATURAS

CAT – catalase

Cr – creatina

DTNB – 5',5-ácido ditio-BIS-nitrobenzóico

EIM – erros inatos do metabolismo

ERN – espécies reativas de nitrogênio

ERO – espécies reativas de oxigênio

GSH – glutationa reduzida

GPx – glutationa peroxidase

H_2O_2 – peróxido de hidrogênio

HL – 3-hidroxi-3-metilglutaril-CoA liase

HMG – ácido 3-hidroxi-3-metilglutárico

HMGA – acidúria 3-hidroxi-3-metiglutárica

HMG-CoA – 3-hidroxi-3-metilglutaril-CoA

ICV – intracerebroventricular

IE – intraestriatal

IP – intraperitoneal

LDL – lipoproteína de baixa densidade

L-NAME – N^{ω}-nitro-L-arginina metil éster

MDA – malondialdeído

MEL – melatonina

MGA – ácido 3-metilglutárico

MGT – ácido 3-metilglutacônico

MGTA – acidúria 3-metilglutacônica

NAC – N-acetilcisteína

$NO^{\bullet}$ – óxido nítrico

8-OHdGA – 8-hidroxi-2'-deoxiguanosina

$O_2^{\bullet -}$ – ânion superóxido

$OH^{\bullet}$ – radical hidroxila

$^1O_2$ – oxigênio singlete

$ONOO^-$ – peroxinitrito

OHIVA – ácido 3-hidroxiisovalérico

PSH – grupamentos sulfidrilas de proteínas

PSSG – proteínas glutationiladas

PSSP – pontes dissulfeto de proteínas

SNC – sistema nervoso central

SOD – superóxido dismutase

TAR – reatividade antioxidante total

TBA-RS – substâncias reativas ao ácido tiobarbitúrico

TRAP – potencial antioxidante total

TRO – trolox

I.1. INTRODUÇÃO

I.1.1. Erros Inatos do Metabolismo

Em 1908, Sir Archibald E. Garrod criou o termo erros inatos do metabolismo (EIM) para designar doenças como a alcaptonúria, em que os indivíduos afetados excretam grandes quantidades de ácido homogentísico na urina. Garrod observou uma maior freqüência desta doença em indivíduos de uma mesma família e maior incidência de consangüinidade entre os pais dos pacientes. Baseando-se nas leis de Mendel e no fato de que os pais dos indivíduos afetados não apresentavam a doença, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da observação de que o ácido homogentísico presente em excesso na urina dos pacientes era um metabólito normal da degradação protéica, ele relacionou este acúmulo a um bloqueio na rota de catabolismo da tirosina. Com o surgimento de novos distúrbios relacionados a alterações genéticas e que envolviam o acúmulo de outras substâncias nos líquidos biológicos dos pacientes, postulou-se que estas doenças resultavam da síntese qualitativa ou quantitativamente anormal de uma proteína, enzimática ou não, pertencente ao metabolismo (Scriver et al., 2001). Presumiu-se então, que em conseqüência deste bloqueio metabólico, pode ocorrer o acúmulo de precursores da reação catalisada pela enzima envolvida, com a formação de rotas metabólicas alternativas e a deficiência de produtos essenciais ao organismo (Bickel, 1987).

Até o momento, foram descritos mais de 500 EIM, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver et al., 2001). Embora individualmente

raras, essas doenças afetam aproximadamente 1 a cada 500-1000 recém nascidos vivos (Baric et al., 2001).

I.1.2. Acidemias 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 freqüência 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 erros inatos do metabolismo, a incidência destas doenças é estimada em 1: 2.200 recém-nascidos, enquanto que, 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 taxa de consangüinidade é elevada, a freqüência é de 1: 740 nascidos vivos (Rashed et al., 1994). Chalmers e colaboradores (1980) demonstraram que as acidemias orgânicas eram os EIM mais freqüentes 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 freqüentes manifestações laboratoriais são cetose, cetonúria, neutropenia, trombocitopenia, acidose metabólica, baixos níveis de bicarbonato, hiperglicinemia, hiperamonemia, hipo / hiperglicemia, acidose láctica, aumento dos níveis séricos de ácidos graxos livres e outros (Scriver et al., 2001). Recentemente, com o uso da tomografia computadorizada, 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.1.3. Acidúria 3-hidroxi-3-metilglutárica

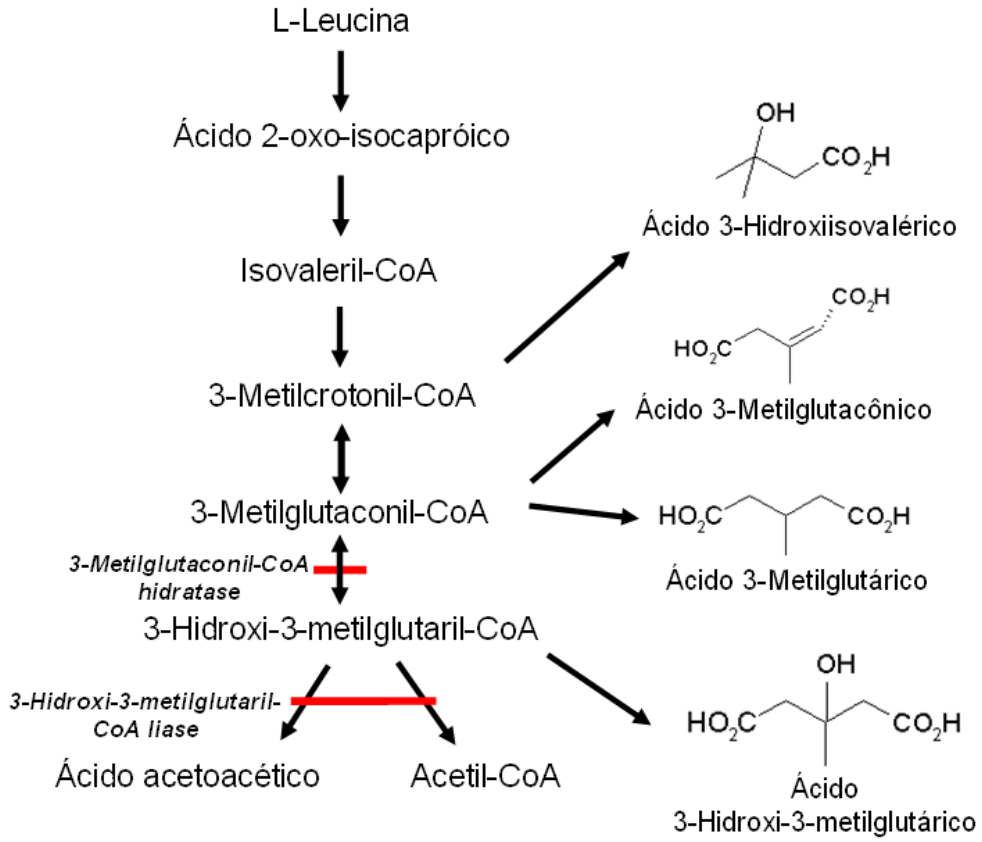
A acidúria 3-hidroxi-3-metilglutárica (HMGA), causada pela deficiência da enzima 3-hidroxi-3-metilglutaril-CoA liase (HL; EC 4.1.3.4), é uma acidúria orgânica de herança autossômica recessiva inicialmente descrita por Faull e colaboradores (1976a; 1976b). A HL catalisa a clivagem do 3-hidroxi-3-metilglutaril-CoA (HMG-CoA) a acetoacetato e acetil-CoA, a qual é a última reação da síntese de corpos cetônicos e do catabolismo da leucina (Figura 1). Estudos demonstram a existência de 90-100 casos de HMGA no mundo, sendo que, com exceção da Arábia Saudita e Península Ibérica (Portugal e Espanha), onde a doença é prevalente, a HMGA é rara em países europeus e no Japão (Vargas et al., 2007; Menao et al., 2009). No Brasil, já foram descritos 15 pacientes portadores da HMGA (Vargas et al., 2007).

Duas isoformas da HL são encontradas, uma localizada na mitocôndria e a outra nos peroxissomos. A HL mitocondrial madura 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 controlados, 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) (Figura I.1). Além disso, durante as crises de descompensação metabólica são detectadas altas concentrações dos ácidos glutárico e adípico. Outros metabólitos importantes encontrados acumulados na urina dos pacientes são as acilcarnitinas (Bonafé et al., 2000; Sweetman e Williams, 2001).



(Figura adaptada de: Sweetman, L. e Williams, J.C., 2001.)

Figura I.1 – Rota do catabolismo da L-Leucina com bloqueio ao nível da enzima 3-metilglutaconil-CoA hidratase e 3-hidroxi-3-metilglutaril-CoA liase.

I.1.3.1. Achados Clínicos e Neuropatológicos

As manifestações clínicas da HMGA se manifestam 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. Enfatize-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.1.3.2. 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 e OHIVA nos tecidos e líquidos biológicos dos pacientes (especialmente na urina) medidos por cromatografia gasosa acoplada à espectrometria de massa. Durante as crises agudas, os ácidos glutárico e adípico e a 3-metilcrotonilglicina também podem estar elevados. A determinação da atividade da HL também é importante para a confirmação do diagnóstico, podendo ser

medida espectrofotometricamente em leucócitos, fibroblastos e plaquetas. O diagnóstico molecular também é útil para a confirmação do diagnóstico (Sweetman e Williams, 2001).

I.1.3.3. Tratamento

A HMGA tem tratamento efetivo disponível. 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 ocorre geralmente durante as crises. A restrição de leucina em 87 mg . Kg^{-1} . d^{-1}, além de diminuição da ingesta de gordura e a administração de L-carnitina (100 mg . Kg^{-1} . d^{-1}) auxilia o desenvolvimento de pacientes com HMGA (Dasouki et al., 1987).

I.1.4. Acidúria 3-metilglutacônica

A acidúria 3-metilglutacônica (MGTA) corresponde a um grupo heterogêneo de doenças metabólicas caracterizado pelo acúmulo dos ácidos 3-metilglutacônico (MGT) e 3-metilglutárico (MGA). Atualmente, são conhecidos cinco tipos distintos de MGTA.

I.1.4.1. Acidúria 3-metilglutacônica (MGTA) tipo I ou deficiência da 3-metilglutaconil-CoA hidratase

A MGTA tipo I (MIM 250950) ou deficiência da enzima 3-metilglutaconil-CoA hidratase (EC 4.2.4.18) é um erro inato do metabolismo da leucina, cujos sintomas variam de mínimos a severos. A enzima mitocondrial 3-metilglutaconil-CoA hidratase é codificada pelo gene *AUH*. O bloqueio

enzimático leva ao acúmulo e elevada excreção urinária do MGT, atingindo concentrações em torno de 500 – 1.000 mmol / mol de creatinina (Gunay-Aygun, 2005). O MGA, derivado da hidrogenação do metilglutaconil-CoA, bem como o ácido 3-hidroxiisovalérico (OHIVA), que é derivado do 3-metilcrotonil-CoA, também se encontram elevados nos tecidos e líquidos biológicos dos pacientes afetados por essa doença (Sweetman e Williams, 2001) (Figura I.1).

I.1.4.1.1. Aspectos Clínicos e Neuropatológicos

Os sinais dessa doença são variados e inespecíficos, incluindo retardo no desenvolvimento psicomotor e neurológico, retardo na linguagem, microcefalia leve, hepatomegalia e distonia, bem como hipoglicemia e acidose metabólica (Shoji et al., 1999; Sweetman e Williams, 2001; Ijlst et al., 2002; Arn e Funanage, 2006). Dentre os achados neuropatógicos, foi observada atrofia dos gânglios basais, incluindo lesões principalmente no globus pallidus e núcleo caudato (Arbelaez et al., 1999). Também já foi evidenciado que tecidos com maior dependência do metabolismo oxidativo, como o sistema nervoso central (SNC) e os músculos esquelético e cardíaco, são predominantemente afetados (Gunay-Aygun, 2005).

I.1.4.1.2. Diagnóstico

O diagnóstico da MGTA I é realizado a partir da análise quantitativa dos ácidos orgânicos na urina, com acúmulo predominante do MGT. A MGTA I tem por característica diferencial ser a única forma de acidúria 3-metilglutacônica onde há acúmulo do OHIVA. O diagnóstico deve ser confirmado medindo-se a atividade da metilglutaconil-CoA hidratase em leucócitos ou fibroblastos. O

diagnóstico pré-natal é possível através do acúmulo do OHIVA e do MGT no líquido amniótico (Sweetman e Williams, 2001).

I.1.4.1.3. Tratamento

O tratamento é realizado com a restrição dietética da leucina e diminuição da ingesta de proteínas. Um estudo evidenciou que a adição de L-carnitina melhorou o estado nutricional e a hepatomegalia nos pacientes afetados pela doença (Gibson et al., 1998).

I.1.4.2. Acidúria 3-metilglutacônica (MGTA) tipo II ou síndrome de Barth

A MGTA II (MIM 302060) ou síndrome de Barth é uma desordem ligada ao cromossomo X caracterizada pelo acúmulo de MGT e MGA e por atividade normal da enzima metilglutaconil-CoA hidratase. É uma doença possivelmente relacionada a um defeito no metabolismo da cardiolipina devido a mutações no gene *TAZ* (tafazina) (Bione et al., 1996), o qual codifica uma aciltranferase envolvida na síntese da cardiolipina. O quadro clínico é caracterizado por cardiomiopatia, neutropenia e miopatia esquelética (Barth et al., 1983; Barth et al., 2004; Arn e Funanage, 2006). A disfunção cardíaca é atribuída à dilatação biventricular e hipertrofia. Também há a deposição de lipídios nos músculos esquelético e cardíaco com diminuição nos níveis de carnitina e anormalidades mitocondriais (Sweetman e Williams, 2001). Além disso, estudos anteriores demonstraram diminuição nas atividades dos complexos I, III, IV e V da cadeia respiratória em músculo esquelético dos pacientes afetados (Barth et al., 1983; Ibel et al., 1993; Christodoulou et al., 1994; Besley et al., 1995; De Kremer et al., 2001).

I.1.4.3. Acidúria 3-metilglutacônica (MGTA) tipo III ou Síndrome da atrofia óptica de Costeff

A MGTA tipo III ou síndrome da atrofia óptica de Costeff (MIM 258501) é relacionada a mutações no gene *OPA3,* caracterizada predominantemente por dano neurológico, que foi detectada inicialmente em indivíduos do Iraque e Israel (Anikster et al., 2001; Arn e Funanage, 2006). Até o presente momento, a função da proteína OPA3 é desconhecida (Davies et al., 2008). Similarmente à MGTA tipo II, apenas o MGT e o MGA se encontram acumulados nos líquidos biológicos e tecidos dos pacientes e a atividade da enzima 3-metilglutaconil-CoA hidratase é normal.

O quadro clínico da MGTA III é caracterizado por atrofia óptica bilateral infantil, coreoatetose, retardo mental, ataxia cerebelar, defeitos cognitivos e outros sintomas neurológicos (Anikster et al., 2001; Sweetman e Williams, 2001; Gunay-Aygun, 2005; Arn e Funanage, 2006).

I.1.4.4. Acidúria 3-metilglutacônica (MGTA) tipo IV ou Não-classificada

A MGTA tipo IV ou Não-classificada (MIM 250951) é composta por um grupo muito heterogêneo de sintomas, incluindo retardo mental e psicomotor, espasticidade, hipertonia, hipotonia, convulsões, atrofia óptica, surdez, cardiomiopatia e disfunção hepática. Os pacientes apresentam elevação nas concentrações de ácido lático e intermediários do ciclo de Krebs, bem como defeitos na cadeia respiratória mitocondrial (Ibel et al., 1993; Besley et al., 1995; Ruesch et al., 1996), tais como diminuição das atividades dos complexos I, II, III, IV e V (Gibson et al., 1992; Sweetman e Williams, 2001; Gunay-Aygun,

2005; Sperl et al., 2006; Wortmann et al., 2006). O MGT e o MGA se encontram predominantemente acumulados na MGTA tipo IV. Por outro lado, a deficiência enzimática que leva a essa condição não é conhecida.

I.1.4.5. Acidúria 3-metilglutacônica (MGTA) tipo V

A MGTA tipo V (MIM 610198) é uma doença autossômica recessiva recentemente descrita por Davey e colaboradores (2006). Essa forma da MGTA foi identificada em populações das planícies do Canadá e do nordeste dos Estados Unidos da América. É caracterizada por cadiomiopatia dilatada, ataxia cerebelar não-progressiva, disgenesia testicular e retardo no crescimento. Em comparação com as outras formas da MGTA, as concentrações de MGT e MGA encontram-se menos aumentadas. Foi sugerido que a mutação responsável por essa desordem ocorre no gene *DNAJC19*, causando defeitos na importação de proteínas mitocondriais (Davey et al, 2006; Wortmann et al., 2009).

I.1.5. Fisiopatologia das acidúrias 3-hidroxi-3-metilgutárica (HMGA) e 3-metilglutacônica (MGTA)

A patogênese desse grupo de distúrbios é desconhecida. No entanto, é possível que os metabólitos acumulados em tecidos e líquidos biológicos dos pacientes afetados por essas doenças sejam tóxicos e responsáveis por pelo menos parte dos sintomas apresentados, embora os mecanismos pelos quais esses ácidos orgânicos possam levar aos sintomas característicos dessas desordens ainda não foram desvendados. Neste particular, um estudo recente

demonstrou que o OHIVA não causa danos sobre o metabolismo energético em cérebro de ratos (Ribeiro et al., 2007).

No que se refere à HMGA, acredita-se que a hipoglicemia que ocorre principalmente durante as crises de descompensação metabólica possa contribuir para a patogênese do dano cerebral nos afetados por essa doença. Acredita-se que a produção insuficiente de glicose devido à inibição da gliconeogênese por HMG-CoA e do elevado consumo de glicose pela falta de corpos cetônicos circulantes possam estar implicados nas alterações neurológicas características da doença. A hipoglicemia associada à diminuição na síntese de corpos cetônicos faz com que o cérebro fique sem substratos energéticos. Nesse contexto, é possível que as lesões na substância branca e outros danos neurológicos encontrados em pacientes afetados pela HMGA ocorram particularmente devido à falta de energia no cérebro. Também foi demonstrado que a hipoglicemia pode inibir a cadeia transportadora de elétrons, causando depleção de ATP, bem como induzir a geração de espécies reativas através da ativação de receptores glutamatérgicos por aumento no influxo de cálcio (Singh et al., 2004; Suh et al., 2007). No entanto, não se pode excluir um efeito neurotóxico dos ácidos acumulados na HMGA. Assim, é possível que a hipoglicemia e a hipocetonemia atuem sinergicamente com os ácidos orgânicos potencialmente neurotóxicos que se acumulam nesta doença, levando ao dano cerebral.

Similarmente, é possível que o acúmulo de MGT e MGA seja importante mecanismo responsável pela fisiopatologia da MGTA. Nesse particular, já foram observadas disfunções mitocondriais em indivíduos portadores das formas II-V da MGTA. Na MGTA tipo II ou síndrome de Barth causada por

mutações no gene *TAZ,* observam-se anormalidades mitocondriais e disfunção na cadeia respiratória (Barth et al., 1983; Ibel et al., 1993; Christodoulou et al., 1994; Besley et al., 1995; De Kremer et al., 2001). O gene *TAZ* codifica a proteína tafazzina, que está envolvida no metabolismo normal da cardiolipina. Esse fosfolipídio tem como principais funções a manutenção da atividade ótima dos complexos enzimáticos da cadeia respiratória e a importação de proteínas para a mitocôndria (MacKenzie et al., 2006). Dessa forma, foi sugerido que os sintomas encontrados nos pacientes portadores da MGTA tipo II podem estar associados com um defeito no metabolismo da cardiolipina. Já a MGTA tipo III ou síndrome de Costeff ocorre devido a mutações no gene *OPA3*. Um estudo publicado recentemente descreve um modelo de camundongos “knockout” para o gene da proteína OPA3, onde os animais homozigotos apresentam características fenotípicas similares às da MGTA tipo III, com perda de células ganglionais da retina e desmielinização dos axônios do nervo óptico causando atrofia óptica, além de disfunção extrapiramidal, espasticidade e ataxia (Davies et al., 2008). Além disso, foi verificado que os camundongos homozigotos apresentaram atividade mitocondrial aumentada, o que poderia ser um mecanismo compensatório para aumentar a produção de energia nos axônios degenerados do nervo óptico. Entretanto, esse aumento na necessidade por energia promove o aumento na produção de espécies reativas na mitocôndria, o que também contribuiria para a degeneração axonal (Davies et al., 2008). Os pacientes acometidos pela MGTA tipo IV ou Não-classificada apresentam, na maioria dos casos, disfunção na fosforilação oxidativa, que leva à diminuição na produção de ATP. Wortmann e colaboradores (2009) demonstraram que 18 pacientes portadores da MGTA tipo IV apresentam defeito na atividade de pelo

menos um dos complexos da cadeia respiratória em biópsia muscular e / ou cultura de fibroblastos. Essa depleção de energia poderia estar envolvida nos danos característicos da MGTA tipo IV. Finalmente, com relação à MGTA tipo V, foi identificada nos pacientes portadores uma mutação no gene *DNAJC19* (Davey et al., 2006). A proteína DNAJC19 é similar a uma proteína identificada em leveduras, a TIM14, que atua como um dos componentes do sistema de importação de proteínas mitocondrial. Dessa forma, um defeito nesse transporte de proteínas possa ser responsável pela condição patológica da MGTA tipo V. Nesse contexto, é importante ressaltar que a síndrome de Mohr-Tranebjaerg, caracterizada por surdez neurosensorial, perda de visão, retardo mental e desordem dos movimentos, era, até então, a única desordem genética causada por um defeito no sistema de importação de proteínas mitocondrial (Tranebjaerg et al., 1995)

I.1.6. Radicais Livres

Um radical livre é qualquer espécie química capaz de existir de forma independente e que contenha um ou mais elétrons desemparelhados (Southorn e Powis, 1988; Halliwell, 2001; Halliwell, 2006; Halliwell e Gutteridge, 2007a).

Em condições fisiológicas do metabolismo celular aeróbico, o oxigênio molecular (O_2) sofre redução tetravalente, com incorporação de quatro elétrons, resultando na formação de água (H_2O). No entanto, aproximadamente 5% do oxigênio utilizado na cadeia respiratória mitocondrial não é completamente reduzido à água, podendo ser convertido a intermediários reativos como o radical superóxido ($O_2^{\bullet -}$) e hidroxila ($OH^{\bullet}$), e também a

peróxido de hidrogênio (H_2O_2), processo esse que pode ser exacerbado em condições patológicas (Boveris, 1998).

O termo genérico “Espécies Reativas de Oxigênio” (ERO) é usado para incluir não só os radicais formados pela redução do O_2, como por exemplo os radicais superóxido ($O_2^{\bullet -}$) e hidroxila ($OH^{\bullet}$), mas também algumas substâncias reativas não-radicais derivados do oxigênio, como o H_2O_2 (Halliwell e Gutteridge, 2007a). Além dessas, existem ainda as espécies reativas de nitrogênio (ERN), sendo o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^-$) os principais representantes.

As ERO e ERN ocorrem tanto em processos fisiológicos quanto patológicos do organismo. Fisiologicamente, essas espécies reativas são importantes para a função celular (Bergendi et al., 1999). Assim, um aumento eventual da liberação local de radicais livres pode ser benéfico, como é o caso da liberação de espécies tóxicas oxidantes pelos neutrófilos que atuam na defesa do hospedeiro contra uma infecção (Delanty e Dichter, 1998; Halliwell e Gutteridge, 2007b). As espécies ativas ainda participam de processos de sinalização celular e também estão envolvidos na síntese e regulação de algumas proteínas (Halliwell e Gutteridge, 2007b).

Por outro lado, quando formadas em excesso, essas espécies altamente reativas têm o potencial de oxidar moléculas (Maxwell, 1995). Com relação aos efeitos prejudiciais das reações oxidantes ao organismo, os radicais livres podem promover lipoperoxidação (oxidação lipídica), causar a oxidação de lipoproteínas de baixa densidade (LDL), reagir com proteínas, levando à sua inativação e consequente alteração de sua função, além de reagir com o DNA

e RNA, levando a mutações somáticas e a alterações na transcrição gênica (Delanty e Dichter, 1998; Halliwell e Whiteman, 2004), dentre outros efeitos.

I.1.6.1. Defesas Antioxidantes

Para evitar os efeitos danosos das espécies reativas, existem mecanismos eficientes para sua eliminação, como a produção endógena de enzimas antioxidantes e alguns antioxidantes não-enzimáticos. Embora diferindo na sua composição, as defesas antioxidantes estão amplamente distribuídas no organismo (Halliwell e Gutteridege, 2007c) e compreendem:

- agentes que removem cataliticamente os radicais livres, como as enzimas superóxido dismutase, catalase e glutationa peroxidase;
- proteínas que diminuem 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 dano oxidativo por outros mecanismos, como as chaperonas;
- agentes de baixo peso molecular que seqüestram espécies reativas de oxigênio e nitrogênio, como glutationa (GSH), α-tocoferol, ácido ascórbico e a bilirrubina;

I.1.6.2. Estresse Oxidativo

Organismos saudáveis em condições normais produzem espécies reativas, que em sua maior parte são controladas pelos sistemas de defesa

antioxidante. No entanto, em determinadas condições patológicas pode haver um desequilíbrio entre a produção de oxidantes e as defesas antioxidantes, favorecendo a ocorrência do estresse oxidativo.

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 que aceleram a produção de algumas espécies reativas, ou então da combinação de quaisquer desses fatores (Halliwell, 2006).

O estresse oxidativo pode promover adaptação, dano ou morte celular:

- Adaptação: as células podem tolerar um estresse oxidativo moderado, que geralmente resulta em um aumento da síntese de sistemas de defesa antioxidante a fim de restaurar o balanço pró-oxidante / antioxidante.
- Dano celular: o estresse oxidativo pode provocar dano a alvos moleculares (DNA, proteínas, carboidratos e lipídios) (Halliwell e Gutteridge, 2007b). Nesses casos, a resposta à injúria tecidual pode ser reversível: a célula entra em um estado de homeostase alterado temporário ou prolongado, que não leva à morte celular.
- 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 GSH, 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 induzi-las a um estresse oxidativo. Já na apoptose, o mecanismo intrínseco de morte celular programada é ativado e não há a liberação do conteúdo celular. A apoptose pode estar acelerada em certas doenças, tais como as desordens neurodegenerativas, havendo envolvimento do estresse oxidativo (Halliwell e Gutteridge, 2007c).

I.1.6.3. Estresse Oxidativo e Doenças Neurodegenerativas

Numerosas hipóteses têm sido propostas para explicar a neurodegeneração das doenças de Alzheimer, Huntington e Parkinson (Alexi et al., 2000; Mendéz-Álvarez et al., 2001; Behl et all., 2002; Halliwell, 2006), sem, entretanto, obter até o momento uma explicação completamente satisfatória para explicar o dano cerebral dessas doenças. No entanto, acredita-se que possíveis mecanismos envolvam deficiência no metabolismo energético, estresse oxidativo e neurotoxicidade mediada por receptores glutamatérgicos do tipo NMDA (excitotoxicidade), ou, possivelmente, um somatório desses fatores (Rose e Henneberry, 1994).

Estudos demonstraram uma diminuição na atividade do complexo I da cadeia respiratória em cérebros *postmortem* de pacientes portadores de doença de Parkinson (Schapira et al., 1990a). Essa inibição do complexo I pode acarretar um aumento 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 a disfunção mitocondrial formem um “ciclo

vicioso” na doença de Parkinson (Schapira et al., 1989, 1990a,b; Janetzky et al., 1994; Gu et al., 1998).

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 e Carney, 1999; Nourooz-Zadeh et al., 1999; Lovell et al., 2000).

Por outro lado, verificou-se um dano oxidativo importante em pacientes portadores da doença de Huntington, particularmente representado pela formação de 3-nitrotirosina nas áreas afetadas (Alexi et al., 2000). Entretanto, o dano oxidativo observado nessa doença aparentemente tem menor importância do que nas doenças de Parkinson e Alzheimer.

Nos últimos anos, foi também verificado que vários metabólitos acumulados em alguns EIM com comprometimento severo do SNC induzem estresse oxidativo no cérebro de animais experimentais (Latini et al., 2007; Ribeiro et al., 2007; Feksa et al., 2008; Kessler et al., 2008; Zugno et al., 2008) e em seres humanos (Sitta et al., 2006; Deon et al., 2007; Barschak et al., 2008a,b; Deon et al., 2008), indicando que os compostos acumulados nestas doenças possam causar dano oxidativo.

I.2. OBJETIVOS

I.2.1. Objetivo Geral

O objetivo do presente trabalho foi verificar os efeitos *in vitro* dos ácidos 3-hidroxi-3-metilglutárico (HMG), 3-metilglutárico (MGA), 3-metilglutacônico (MGT) e 3-hidroxiisovalérico (OHIVA) sobre parâmetros de estresse oxidativo em córtex cerebral, estriado e fígado de ratos jovens. O estudo visou a uma melhor compreensão dos mecanismos tóxicos desses metabólitos acumulados nas acidúrias 3-hidroxi-3-metilglutárica e 3-metilglutacônica.

I.2.2. Objetivos Específicos

a) Determinar os efeitos *in vitro* dos ácidos HMG, MGA, MGT e OHIVA sobre a peroxidação lipídica, medida através das substâncias reativas ao ácido tiobarbitúrico (TBA-RS) e da quimioluminescência, em córtex cerebral, estriado e fígado de ratos de 30 dias de vida.

b) Determinar os efeitos *in vitro* dos ácidos HMG, MGA, MGT e OHIVA sobre a oxidação de proteínas, determinada através da formação de carbonilas e da medida de grupamentos sulfidrila em córtex cerebral, estriado e fígado de ratos de 30 dias de vida.

c) Determinar os efeitos *in vitro* dos ácidos HMG, MGA, MGT e OHIVA sobre as defesas antioxidantes não enzimáticas, medidas pelo conteúdo de glutationa reduzida (GSH), potencial antioxidante total (TRAP) e reatividade antioxidante total (TAR) em córtex cerebral, estriado e fígado de ratos de 30 dias de vida.

d) Determinar os efeitos *in vitro* dos ácidos HMG, MGA, MGT e OHIVA sobre a oxidação de uma solução comercial de GSH na ausência de qualquer tecido.

e) Determinar os efeitos *in vitro* dos ácidos HMG, MGA, MGT e OHIVA sobre as atividades das enzimas antioxidantes superóxido dismutase (SOD),

glutationa peroxidase (GPx) e catalase (CAT) em córtex cerebral de ratos de 30 dias de vida.

PARTE II

Artigos Científicos

Capítulo I

Evidence that 3-hydroxy-3-methylglutaric acid promotes lipid and protein oxidative damage and reduces the nonenzymatic antioxidant defenses in rat cerebral cortex

Guilhian Leipnitz, Bianca Seminotti, Josué Haubrich, Manuela B. Dalcin, Karina B. Dalcin, Alexandre Solano, Giorgia de M. de Bortoli, Rafael B. Rosa, Alexandre U. Amaral, Carlos S. Dutra-Filho, Alexandra Latini, Moacir Wajner.

Artigo científico publicado no periódico

Journal of Neuroscience Research

Journal of Neuroscience Research 86:683–693 (2008)

Evidence That 3-Hydroxy-3-Methylglutaric Acid Promotes Lipid and Protein Oxidative Damage and Reduces the Nonenzymatic Antioxidant Defenses in Rat Cerebral Cortex

Guilhian Leipnitz,[1] Bianca Seminotti,[1] Josué Haubrich,[1] Manuela B. Dalcin,[1] Karina B. Dalcin,[1] Alexandre Solano,[1] Giorgia de Bortoli,[1] Rafael B. Rosa,[1] Alexandre U. Amaral,[1] Carlos S. Dutra-Filho,[1] Alexandra Latini,[2] and Moacir Wajner[1–4]★

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul, Porto Alegre, RS, Brazil
[2]Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[3]Hospital de Clínicas, Serviço de Genética Médica, Porto Alegre, RS, Brazil
[4]Universidade Luterana do Brasil, Canoas, Brazil

In the present work we investigated the in vitro effect of 3-hydroxy-3-methylglutarate (HMG) that accumulates in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency (HMGLD) on important parameters of oxidative stress in rat cerebral cortex. It was observed that HMG induced lipid peroxidation by significantly increasing chemiluminescence and levels of thiobarbituric acid-reactive substances (TBA-RS). This effect was prevented by the antioxidants α-tocopherol, melatonin, N-acetylcysteine, and superoxide dismutase plus catalase, suggesting that free radicals were involved in the lipid oxidative damage. On the other hand, HMG did not change TBA-RS levels in intact or disrupted mitochondrial preparations, indicating that generation of oxidants by this organic acid was dependent on cytosolic mechanisms. HMG also induced protein oxidative damage in cortical supernatants, which was reflected by increased carbonyl content and sulfhydryl oxidation. Furthermore, HMG significantly reduced the nonenzymatic antioxidant defenses total-radical trapping antioxidant potential, total antioxidant reactivity, and reduced glutathione (GSH) levels in rat cerebral cortex. HMG-induced GSH reduction was totally blocked by melatonin pretreatment. We also verified that the decrease of GSH levels provoked by HMG in cortical supernatants was not due to a direct oxidative effect of this organic acid, because exposition of commercial GSH and purified membrane protein-bound thiol groups to HMG in the absence of cortical supernatants did not decrease the reduced sulfhydryl groups. Finally, the activities of the main antioxidant enzymes were not altered by HMG exposure. Our data indicate that oxidative stress elicited in vitro by HMG may possibly contribute at least in part to the pathophysiology of the brain injury in HMGLD.

Key words: 3-hydroxy-3-methylglutaric acid; 3-hydroxy-3-methylglutaric aciduria; oxidative stress

3-Hydroxy-3-methylglutaric aciduria (HMGA; Mckusick 246450) is an autossomal recessive neurometabolic disorder of leucine catabolism and of ketogenesis caused by 3-hydroxy-3-methylglutaryl-CoA lyase deficiency (HMGLD; Faull et al., 1976; Wysocki et al., 1976). This mitochondrial and peroxissomal enzyme catalyzes the cleavage of 3-hydroxy-3-methylglutaril-CoA to acetoacetate acid and acetyl-CoA. The disorder is biochemically characterized by predominant tissue accumulation and high urinary excretion of large quantities of 3-hydroxy-3-methylglutarate (HMG), in addition to 3-methylglutaconate, 3-methylglutarate, 3-hydroxyisovalerate, and 3-methylcrotonylglycine (Bonafé et al., 2000; Sweetman et al., 2001). The average concentration of urinary HMG in HMGA patients is about 1,300 mmol/mol of creatinine, but it can rise to 11,000 mmol/

Contract grant sponsor: FAPERGS; Contract grant sponsor: PRONEX II; Contract grant sponsor: CNPq-Brazil.

★Correspondence to: Moacir Wajner, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul. Rua Ramiro Barcelos No. 2600 Anexo, CEP 90035-003, Porto Alegre, RS, Brasil. E-mail: mwajner@ufrgs.br

Received 18 May 2007; Revised 19 July 2007; Accepted 26 July 2007

Published online 16 October 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21527

mol of creatinine during metabolic crises (Sweetman et al., 2001). Smaller, but appreciable, levels of glutarate, lactate, adipate, and other dicarboxylic acids may also be excreted in the urine, especially during metabolic decompensation (Gibson et al., 1988a,b).

Considerable heterogeneity has been observed in clinical and biochemical HMGLD presentation. The cardinal clinical features are similar to those of Reye's syndrome and include acute episodes with vomiting, hepatomegaly, hypotonia, and lethargy that progress to coma (Gibson et al., 1988a,b; Lee et al., 1999; Funghini et al., 2001). Other symptoms are macrocephalia, developmental delay, and dilated cardiomiopathy (Gibson et al., 1994; Funghini et al., 2001). The main laboratory findings during crises are hypoglycemia, variable hyperammonemia, metabolic acidosis, and elevated transaminases without ketosis. About 30% of HMGLD patients become symptomatic in the neonatal period and about 60% between 3 and 12 months in the early infantile period. In the neonatal period, the disease may lead to a fatal outcome unless promptly treated (Lyon et al., 1996).

The most common abnormalities found in the magnetic resonance neuroimaging scanning involve multiple and marked coalescent lesions in periventricular subcortical white matter and arcuate fibers, most prominently in frontal or periatrial regions. Involvement of the caudate nucleus and the dentate nucleus is also observed in HMGLD patients (van der Knaap et al., 1998; Yalcinkaya et al., 1999; Yylmaz et al., 2006).

Although HMGLD-affected patients present severe neurological symptoms, the underlying mechanisms of brain damage in this disorder are not yet established. Therefore, in the present work, we investigated the in vitro effect of HMG, the major metabolite accumulating in HMGA, on a wide spectrum of oxidative stress parameters, namely, thiobarbituric acid-reactive substances (TBA-RS), chemiluminescence, total-radical trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), glutathione (GSH) levels, carbonyl content, sulfhydryl oxidation, superoxide generation, and activities of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) in cerebral cortex of young rats to evaluate whether oxidative stress could be involved in the pathophysiology of the neurological damage in HMGLD. We also tested the role of antioxidants on the effects elicited by HMG.

MATERIALS AND METHODS

Animals and Reagents

Thirty-day-old 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-hr light/dark cycle (lights on 0700–1900 hr) in an air-conditioned constant-temperature (22°C ± 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 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.

All chemicals were purchased from Sigma (St. Louis, MO). HMG was prepared on the day of the experiments in the incubation medium used for each technique, and pH was adjusted to 7.4. The final concentrations of the acid in the medium ranged from 0.1 to 5.0 mM. Controls did not contain the metabolite in the incubation medium. In some experiments, antioxidants were added to the incubation medium at the following final concentrations: 1.5 or 5 μM trolox (TRO), 200 or 1500 μM melatonin (MEL), combination of SOD plus CAT (2.5 or 10 mU/ml each), 100 μM GSH, 500 μM N^{ω}-nitro-L-arginine (L-NAME), 1.0 mM N-acetylcysteine (NAC), and 3.0 mM creatine (Cr). Chemiluminescence, TRAP, and TAR were assayed by using a Wallac scintillation counter. TBA-RS, carbonyl content, superoxide levels, and the antioxidant enzyme activities were measured with a double-bean Hitachi U-2001 spectrophotometer with temperature control. GSH levels were measured in a Hitachi F-2000 fluorescence spectrophotometer.

Cerebral Cortex Supernatant Preparation

On the day of the experiments, the rats were sacrificed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum, and striatum were discarded, and the cerebral cortex was dissected, weighed, and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750*g* for 10 min at 4°C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated at 37°C for 1 hr with HMG. In some experiments, we used a 15-min preincubation with antioxidants before the addition of HMG. Immediately after incubation, aliquots were taken to measure TBA-RS, chemiluminescence, TRAP, TAR, GSH concentrations, carbonyl content, sulfhydryl oxidation, and the activities of CAT, GPx, and SOD.

Preparation of Mitochondrial Fractions

Mitochondrial fractions were prepared from cerebrum (total brain excluding cerebellum, olfactory bulbs, pons, and medulla) of 30-day-old rats. The cerebrum was homogenized in 10 volumes of 5 mM potassium phosphate buffer, pH 7.4, containing 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA, and 0.1% bovine serum albumin. The homogenate was centrifuged at 1,500*g* for 10 min at 4°C, and the pellet was discarded. The supernatant was then centrifuged for a further 10 min at 4°C at 15,000*g* to isolate the mitochondria present in the pellet, which was finally suspended in the same buffer. Intact and disrupted mitochondrial fractions obtained by freezing/thawing three times were incubated at 37°C for 1 hr with HMG at concentrations of 2.5 and 5 mM. Immediately after incubation, aliquots were used to measure TBA-RS.

Mitochondrial Membrane Preparation

Cerebral cortex was homogenized in 10 mM Tris, pH 7.4, containing 0.25 M sucrose, and the homogenates were centrifuged at 650*g* for 10 min to discard nuclei and cell debris (Kowaltowski et al., 1997). After a new centrifugation at 25,000*g* for 15 min, the resulting pellet containing the mitochondria was suspended in 0.1 M Tris, pH 8.0, and stored in aliquots at −70°C. On the day of the experiment, the aliquots of purified mitochondrial fractions were frozen/thawed three times and centrifuged at 15,000*g* for 2 min to separate the mitochondrial membranes. The resulting pellet was washed with 6.5% TCA three times and centrifuged at 15,000*g* for 2 min. The final pellet was suspended in 0.5 M Tris buffer, pH 8.3, containing 0.5 mM EDTA and used to measure the mitochondrial membrane sulfhydryl (thiol)-bound content.

Isolation of Submitochondrial Particles

Submitochondrial particles were prepared at 4°C from frozen and thawed mitochondria (20 mg protein/ml) according to Poderoso et al. (1996). The obtained submitochondrial particles were washed twice with 140 mM KCl, 20 mM Tris-HCl, pH 7.4, and suspended in the same medium. The suspended particles were then incubated at 37°C for 1 hr with 5 mm HMG. Immediately after incubation, aliquots from this preparation were used to measure superoxide formation.

Superoxide Content

Superoxide production was determined spectrophotometrically according to Poderoso et al. (1996) after exposing submitochondrial particles to HMG. The assay is based on superoxide-dependent oxidation of epinephrine to adenochrome at 37°C (E_{480nm} = 4.0 mM/cm). The reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, pH 7.4, 0.1 mM catalase, 1 mM epinephrine, and 7 mM succinate. SOD was used at 0.1–0.3 mM final concentrations as a negative control to confirm assay specificity.

TBA-RS

TBA-RS level was determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μl of cold 10% trichloroacetic acid was added to 150 μl of HMG pretreated cerebral cortex supernatants and centrifuged at 300*g* for 10 min. Three hundred microliters of the supernatants was transferred to a pyrex tube and incubated with 300 μl of 0.67% TBA in 7.1% sodium sulfate in a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool in running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol/mg protein and are presented as percentage of control.

Chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room by the method of Gonzalez-Flecha et al. (1991). Incubation flasks containing 3.5 ml of 20 mM sodium phosphate buffer, pH 7.4, with 140 mM KCl were counted for background chemiluminescence during 5 min. An aliquot of 500 μl cortical supernatants was immediately added, and chemiluminescence was measured for 30 min at room temperature. The background chemiluminescence was subtracted from the final luminescence value, and the results were calculated as cpm/mg protein and represented as percentage of control.

Determination of Protein Carbonyl Content

Protein carbonyl formation (PCF), a marker of protein oxidative damage, was measured spectrophotometrically according to Reznick and Packer (1994). One hundred microliters of the aliquots from the incubation was treated with 400 μl of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 hr. Samples were then precipitated with 500 μl 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 6 M guanidine prepared in 2.5 N HCl at 37°C for 5 min. The difference between the DNPH-treated and the HCl-treated samples (blank) was used to calculate the carbonyl content determined at 365 nm. The results were calculated as nmol of carbonyls groups/mg protein and are presented as percentage of control, using the extinction coefficient of 22,000 × 10^6 nmol/ml for aliphatic hydrazones.

TRAP

TRAP, representing the total nonenzymatic antioxidant capacity of the tissue, was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane; ABAP) according to the method of Lissi et al. (1992). The reaction mixture containing 4 ml 10 mM ABAP dissolved in 0.1 M glycine buffer, pH 8.6, and 10 μl luminol (4 mM) generates, at room temperature, an almost constant light intensity corresponding to free radical formation, which was measured in a Wallac 1409 liquid scintillation counter. This was considered to be the initial chemiluminescence values. Then, 10 μl of 300 μM trolox (soluble α-tocopherol analogue) or 50 μl of cortical supernatants was added to the reaction medium. The addition of trolox or supernatants provokes a marked reduction of the light intensity, which is maintained for a certain period, after which light intensity rapidly increases. This period corresponds to induction time (IT) and represents TRAP measurement. IT is directly proportional to the antioxidant capacity of the tissue, and the IT of each sample was compared with the IT of trolox. TRAP values were calculated as nmol trolox/mg of protein and are expressed as percentage of control.

TAR

TAR, which represents the quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by ABAP according to the method of Lissi et al. (1995). The chemiluminescence value was measured after 1 min following addition of 4 ml 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) and 10 μl of luminol into a glass scintillation vial (initial chemiluminescence). Ten microliters of 10–100 μM trolox (calibration curve) or brain supernatants, which decrease light intensity, was then added, and chemiluminescence was measured after 60 sec (final chemilu-

minescence). The ratio between the initial and the final chemiluminescence values was used to calculate TAR. TAR values are expressed as nmol trolox/mg of protein and are presented as percentage of control.

Reduced GSH Content

GSH concentrations were measured according to Browne and Armstrong (1998). HMG-pretreated cerebral cortex supernatants were diluted in 20 volumes of (1:20, v/v) 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation was incubated with an equal volume of o-phthaldialdehyde (1 mg/ml methanol) at room temperature over 15 min. Fluorescence was measured by using excitation and emission wavelengths of 350 nm and 420 nm, respectively. A calibration curve was prepared with standard GSH (0.01–1 mM), and the concentrations were calculated as nmol/mg protein and are presented as percentage of control.

We also tested whether HMG could oxidize a commercial solution of GSH (200 μM) by exposing this solution to 5.0 mM HMG for 60 min in a medium devoid of brain supernatants. N-ethylmaleimide (250 μM), a classical oxidant of sulfhydryl groups, was used as a positive control. After HMG exposition, 7.4 mM o-phthaldialdehyde (1 mg/ml) was added to the vials, and the mixture was incubated at room temperature over 15 min.

Sulfhydryl Content

This assay is based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid; DTNB) by thiols, generating a yellow derivative (TNB), whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). *p*-Hydroxymercuribenzoic acid (250 μM) was used as a positive control. Briefly, 40 μl mitochondrial membrane suspension or 120 μl cortical supernatant was incubated at 37°C for 1 hr with HMG at concentrations of 0.1, 1, 2.5, and 5 mM. Then, 1 ml of 0.1 mM DTNB was added. This was followed by a 30-min incubation at room temperature in a dark room. Absorption was measured at 412 nm. The protein-bound sulfhydryl content is inversely correlated to oxidative damage to proteins. Results are reported as nmol TNB/mg protein and are presented as percentage of control.

Antioxidant Enzymes

CAT activity. CAT activity was assayed according to Aebi (1984) by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.1–0.3 mg protein/ml. One unit (U) of the enzyme is defined as 1 μmol H_2O_2 consumed per minute. The specific activity was calculated as U/mg protein and is depicted as percentage of control in cerebral cortex supernatants.

GPx activity. GPx activity was measured according to Wendel (1981) using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/ethylenediaminetetraacetic acid 1 mM, pH 7.7, 2 mM glutathione, 0.15 U/ml glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH, and the supernatant containing 0.2–0.3 mg protein/ml. One GPx unit (U) is defined as 1 μmol NADPH consumed per minute. The specific activity was calculated as U/mg protein and is expressed as percentage of control in cerebral cortex supernatants.

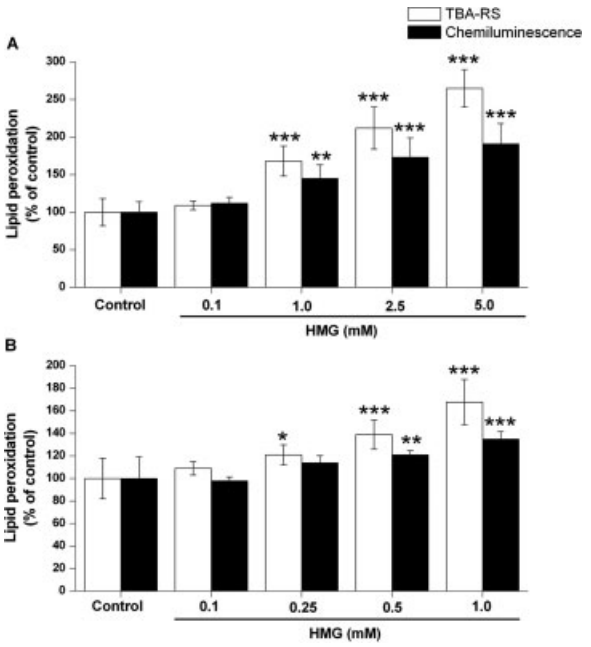


Fig. 1. In vitro effect of 3-hydroxy-3-methylglutarate (HMG) on the lipid peroxidation parameters chemiluminescence and thiobarbituric acid-reactive substances (TBA-RS) in rat cerebral cortex supernatants. Cortical supernatants were incubated for 60 min in the presence of HMG at concentrations ranging from 0.1 to 5.0 mM (**A**) and from 0.1 to 1.0 mM (**B**). Values are means ± standard deviation for five or six independent experiments performed in triplicate and are expressed as percentage of controls (A: controls: chemiluminescence 4,442 ± 624 cpm/mg protein, TBA-RS levels 3.42 ± 0.62 nmol/mg protein; B: controls: chemiluminescence 4,295 ± 823 cpm/mg protein, TBA-RS levels 3.42 ± 0.62 nmol/mg protein). $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$ compared with controls (Duncan multiple range test).

SOD activity. SOD activity was assayed according to Marklund (1985) and is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O_2^-, which is a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. A calibration curve was performed with purified SOD as standard, to calculate the activity of SOD present in the samples. The results are reported as U/mg protein and are expressed as percentage of control in cerebral cortex supernatants.

Protein Determination

Protein content was determined in cerebral cortex supernatants by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

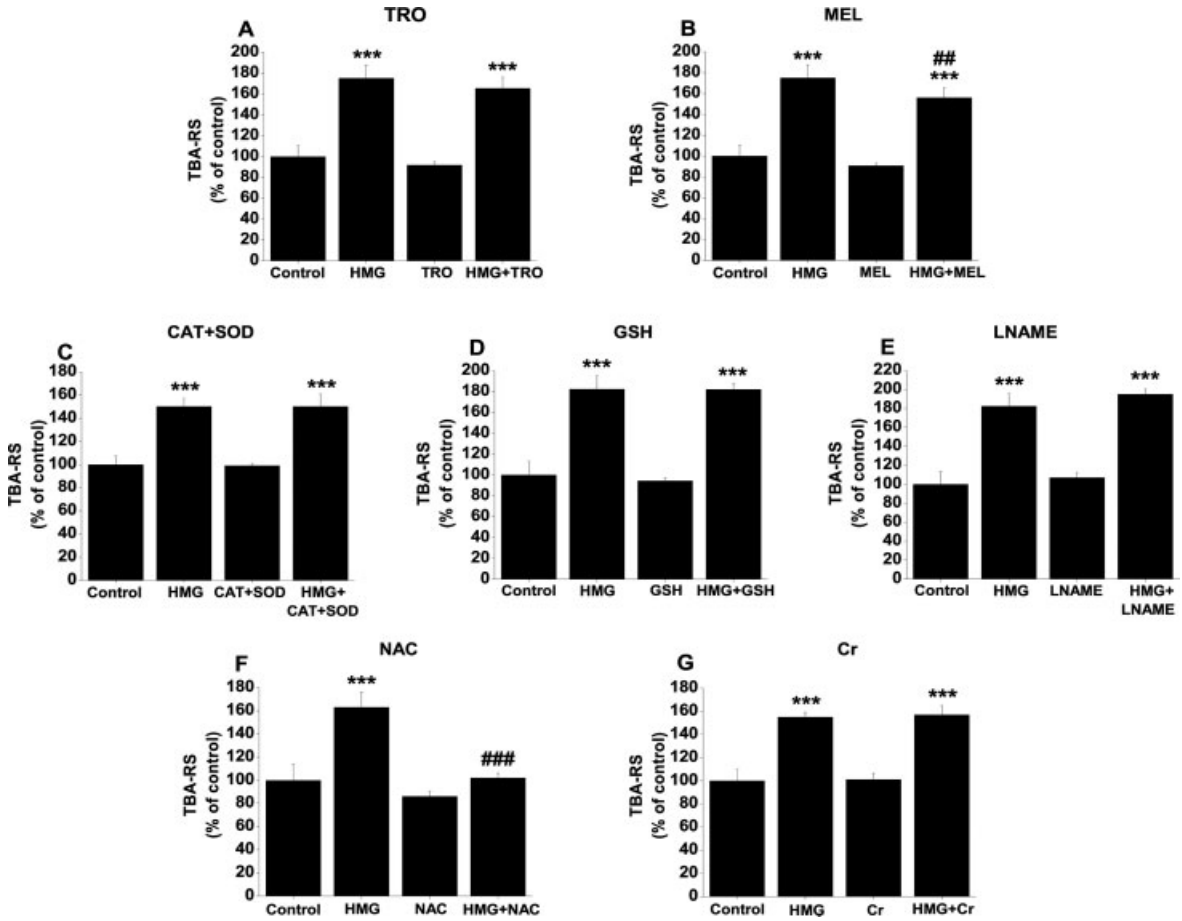


Fig. 2. Effect of antioxidants on 3-hydroxy-3-methylglutarate (HMG)-induced in vitro lipid peroxidation (TBA-RS) in rat cerebral cortex supernatants. Cortical supernatants were preincubated for 15 min with 1.5 μM Trolox (TRO; **A**), 200 μM melatonin (MEL; **B**), a combination of 2.5 mU/ml catalase (CAT) plus 2.5 mU/ml superoxide dismutase (SOD; **C**), 100 μM glutathione (GSH; **D**), 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME; **E**), 1.0 mM N-acetylcysteine (NAC; **F**) or 3.0 mM creatine (Cr; **G**) before the addition of 1 mM HMG. Values are means ± standard deviation for four or five independent experiments performed in triplicate and are expressed as percentage of controls [controls (nmol/mg protein): TRO: 5.38 ± 0.57; MEL: 5.38 ± 0.57; CAT + SOD: 4.93 ± 0.36; GSH: 3.55 ± 0.47; L-NAME: 3.55 ± 0,47; NAC: 5.62 ± 0.80; Cr: 2.64 ± 0.22]. ***$P < 0.001$ compared with controls; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with 1.0 mM HMG (Duncan multiple range test).

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by the Duncan multiple range test when the F value was significant. Linear regression analysis was also used to test dose-dependent effects. Only significant F values are shown in the text. All analyses were performed in the Statistical Package for the Social Sciences (SPSS) software on a PC-compatible computer. $P < 0.05$ was considered significant.

RESULTS

HMG Induces Lipid and Protein Oxidation in Rat Cortical Supernatants

Figure 1 shows that chemiluminescence values were significantly increased (up to 91%) in cortical supernatants exposed to HMG at concentrations of 0.5 mM and higher [A: $F(4,25) = 22.15$, $P < 0.001$; B: $F(1,8) = 39.64$, $P < 0.001$] in a dose-dependent manner (A: $\beta = 0.83$, $P < 0.001$; B: $\beta = 0.829$, $P < 0.001$). TBA-RS levels were also markedly augmented (up to 165%) by HMG in cortical supernatants exposed to 0.25 mM and higher doses of this organic acid [A: $F(4,20) = 207.94$, $P < 0.001$; B: $F(4,20) = 55.16$, $P < 0.001$] in a concentration-dependent manner (A: $\beta = 0.94$, $P < 0.001$; B: $\beta = 0.878$; $P < 0.001$; Fig. 1).

Next, we evaluated the role of antioxidants in HMG-induced increase of TBA-RS levels. Cortical supernatants were preincubated for 15 min at 37°C with the antioxidants TRO (α-tocopherol; 1.5 μM), MEL (200 μM), the combination of SOD and CAT (2.5 mU/ml each), GSH (100 μM), L-NAME (500 μM), NAC (1.0 mM), or Cr (3.0 mM) before the addition of 1.0 mM HMG. It can be observed that, at these concentrations, the antioxidants, except for MEL [$F(3,16) = 92.97$; $P < 0.001$] and NAC [$F(3,12) = 48.309$; $P < 0.001$], were not able to prevent HMG in-vitro-elicited

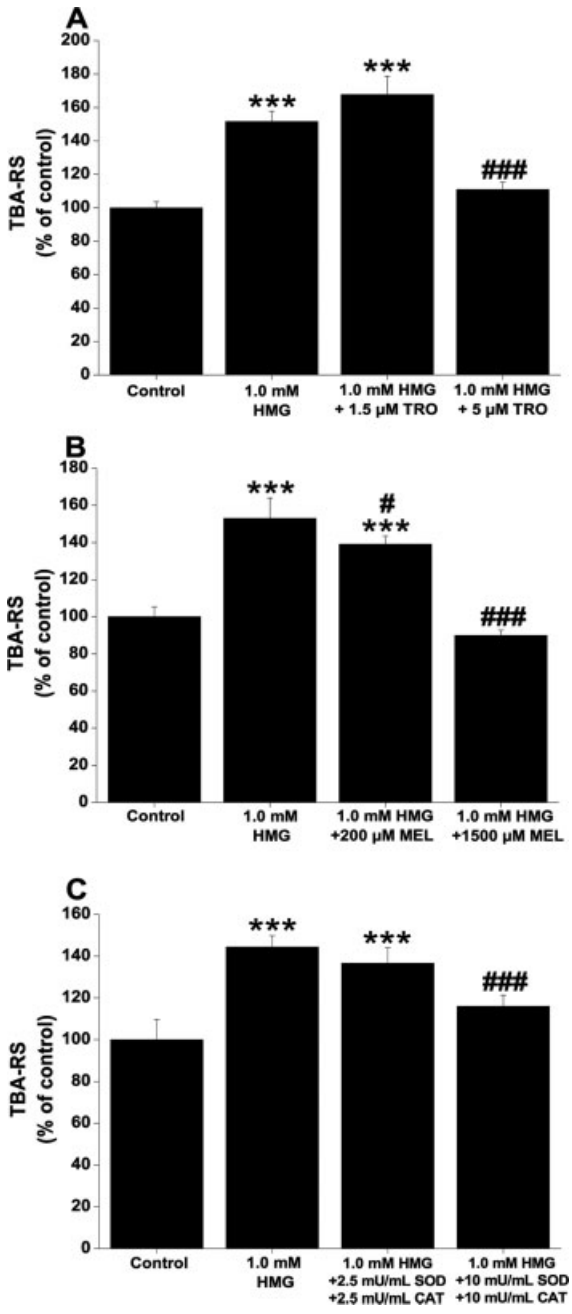


Fig. 3. Effect of increasing concentrations of antioxidants on 3-hydroxy-3-methylglutarate (HMG)-induced in vitro lipid peroxidation (TBA-RS) in rat cerebral cortex supernatants. Cortical supernatants were preincubated for 15 min with the antioxidants Trolox (TRO; 1.5 or 5 μM; **A**), melatonin (MEL; 200 or 1,500 μM; **B**), or a combination of catalase (CAT) plus superoxide dismutase (SOD; 2.5 or 10 mU/ml of each enzyme; **C**) before the addition of 1.0 mM HMG. Values are means ± standard deviation of five independent experiments performed in triplicate and are expressed as percentage of controls [controls (nmol/mg protein): TRO: 5.42 ± 0.2; MEL: 4.29 ± 0.23; SOD + CAT: 3.96 ± 0.38]. ***$P < 0.001$ compared with controls; $^{\#}P < 0.01$, $^{\#\#\#}P < 0.01$ compared with 1.0 mM HMG (Duncan multiple range test).

lipid peroxidation (Fig. 2). However, Figure 2 also shows that MEL attenuated (up to 19% decrease) and NAC totally blocked the lipid oxidative damage induced by HMG. Thereafter, we tested whether higher doses of the antioxidants could prevent the augmented levels of TBA-RS. Cortical supernatants were preincubated for 15 min with TRO (1.5–5 μM), MEL (200–1,500 μM), or the combination of SOD and CAT (2.5–10 mU/ml each) before the addition of 1.0 mM HMG. The results show that, at the higher doses, all tested antioxidants (TRO, MEL, and SOD plus CAT) totally prevented the lipid peroxidation induced by HMG [TRO: $F(3,12) = 87.87$; $P < 0.001$; MEL: $F(3,12) = 83.73$; $P < 0.001$; SOD + CAT: $F(3,12) = 32.07$; $P < 0.001$; Fig. 3].

We also verified that concentrations as high as 5.0 mM of HMG did not change TBA-RS levels in intact and disrupted mitochondrial preparations, indicating that generation of oxidants by this organic acid causing lipid damage occurred via cytosolic rather than mitochondrial mechanisms (results not shown). Protein oxidative damage was also assessed by measuring the carbonyl (Fig. 4A,B) and sulfhydryl content (Fig. 4C) in HMG-treated cortical supernatants. Figure 4 shows that HMG provoked a highly significant increase of carbonyl formation (up to 235%) at concentrations as low as 0.5 mM [A: $F(4,17) = 51.78$, $P < 0.001$; B: $F(4,30) = 5.012$; $P < 0.01$] in a dose-dependent way (A: $\beta = 0.94$, $P < 0.001$; B: $\beta = 0.63$). Furthermore, we observed that HMG induced sulfhydryl oxidation (up to 48% decrease of reduced sulfhydryl content) at 1 mM and higher concentrations [$F(4,16) = 12.55$; $P < 0.001$] in a dose-dependent fashion ($\beta = -0.804$, $P < 0.001$; Fig. 4C).

HMG Reduces the Nonenzymatic Antioxidant Defenses in Rat Cortical Supernatants

The effect of HMG on the nonenzymatic antioxidant defenses, assessed by TRAP, TAR, and GSH measurements, were then investigated. Figure 5 shows that TRAP (Fig. 5A) and TAR (Fig. 5B) measurements were significantly reduced (up to 34%) in cerebral cortex supernatants at HMG concentrations as low as 1 mM [$F(4,29) = 4.34$; $P < 0.01$; Fig. 5A,B]. Figure 5 also shows that HMG treatment provoked a marked reduction of GSH concentrations (up to 50%) in a dose-dependent way [$F(4,19) = 5.21$; $P < 0.01$; $\beta = 0.69$; $P < 0.01$; Fig. 5C]. We also tested whether TRO, MEL, and SOD plus CAT could prevent the HMG decrease of GSH levels in cortical supernatants. We observed that melatonin [$F(4,15) = 82.89$; $P < 0.001$], but not TRO or SOD plus CAT, was able to prevent the HMG decrease of reduced GSH levels in cortical supernatants (Fig. 5D). We also found that the reduction of HMG-induced GSH levels (major naturally occurring nonenzymatic antioxidant defense) was inversely correlated with TBA-RS values (a lipid peroxidation parameter; $\beta = -0.757$; $P < 0.01$), suggesting that free radicals inducing lipid peroxidation were responsible for GSH consumption.

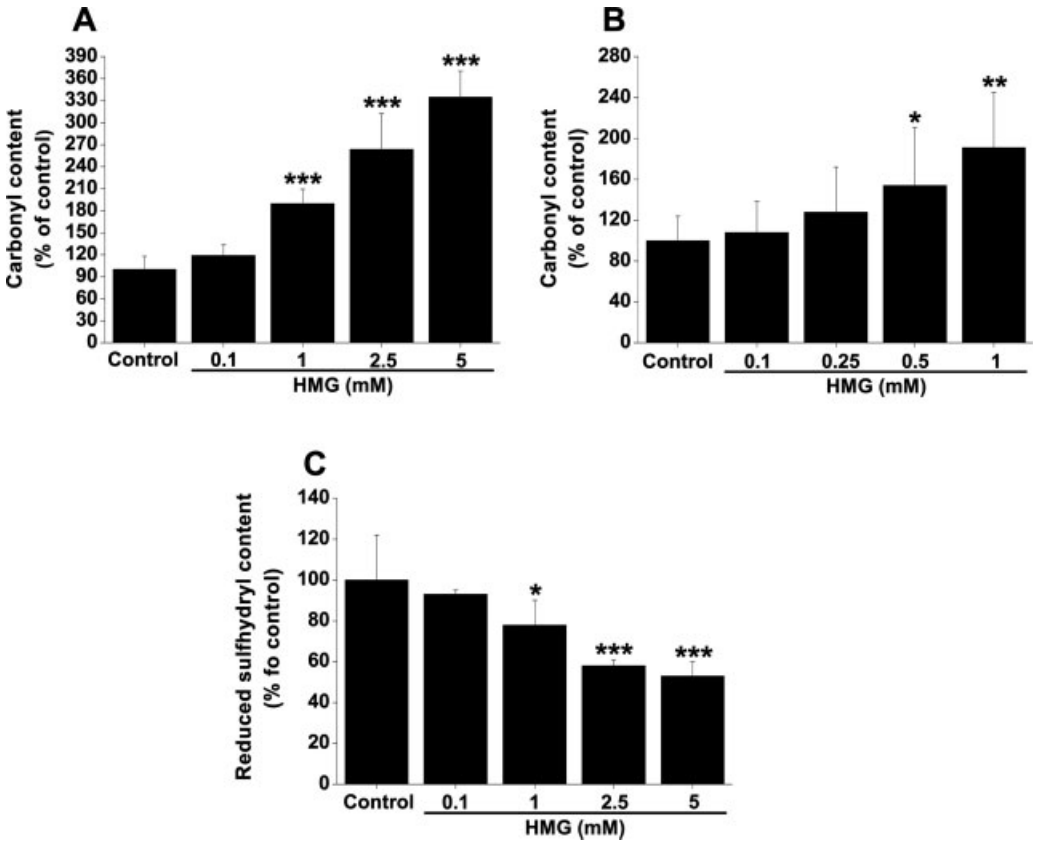


Fig. 4. In vitro effect of 3-hydroxy-3-methylglutarate (HMG) on carbonyl (**A,B**) and reduced sulfhydryl (**C**) content in rat cerebral cortex supernatants. Cortical supernatants were incubated during 60 min in the presence of HMG at concentrations ranging from 0.1 to 5.0 mM. Values are means ± standard deviation for five to seven independent experiments performed in triplicate and are expressed as percentage of controls (A: control: 0.68 ± 0.12 nmol/mg of protein; B: control: 0.52 ± 0.12 nmol/mg of protein; C: control: 56.4 ± 6.8 nmol/mg of protein). $\star P < 0.05$, $\star\star P < 0.01$, $\star\star\star P < 0.001$ compared with controls (Duncan multiple range test).

HMG Per Se Does Not Behave as a Direct Oxidant

The next set of experiments was designed to investigate whether the HMG-induced decrease in brain GSH levels was due to a direct oxidative attack rather than to promotion of free radicals. We therefore exposed commercial GSH and brain purified mitochondrial membrane protein-bound sulfhydryl groups (PBSG) to 5.0 mM HMG in the absence of tissue supernatants. Table I shows that HMG per se did not oxidize free GSH or PBSG, whereas *N*-ethylmaleimide (NEM; 250 μM) and *p*-hydroxymercuribenzoic acid (PHMB; 250 μM) exposition (positive controls) markedly oxidized GSH and PBSG, respectively. The data indicate that HMG itself does not behave as an oxidant agent.

HMG Stimulates Superoxide Generation in Rat Cerebral Cortex

We also tested the effect of 5 mM HMG exposure for 1 hr on superoxide generation in submitochondrial particles obtained from cerebral cortex supernatants. We observed that HMG provoked a significant increase (50%) of superoxide content [$t(4) = 3.332$; $P < 0.05$; Fig. 6].

HMG Does Not Modify the Activities of the Antioxidant Enzymes in Rat Cortical Supernatants

Finally, the effect of HMG on the activity of the antioxidant enzymes CAT, GPx, and SOD was investigated. For this purpose, we measured these enzyme activities after 60 min of incubation of cortical supernatants in the presence of 2.5 or 5.0 mM HMG. Table II shows that these activities were not altered by the metabolite.

DISCUSSION

Individuals affected by 3-hydroxy-3-methylglutaryl-CoA lyase deficiency (HMGLD) accumulate 3-hydroxy-3-methylglutaric acid (HMG) predominantly in their tissues and body fluids. The concentrations of this metabolite dramatically increase during acute metabolic crises, which are clinically characterized by severe hypoketotic hypoglycaemia, acidosis, hyperammonaemia, vomiting, hypotonia, coma, and seizures (Wysocki and

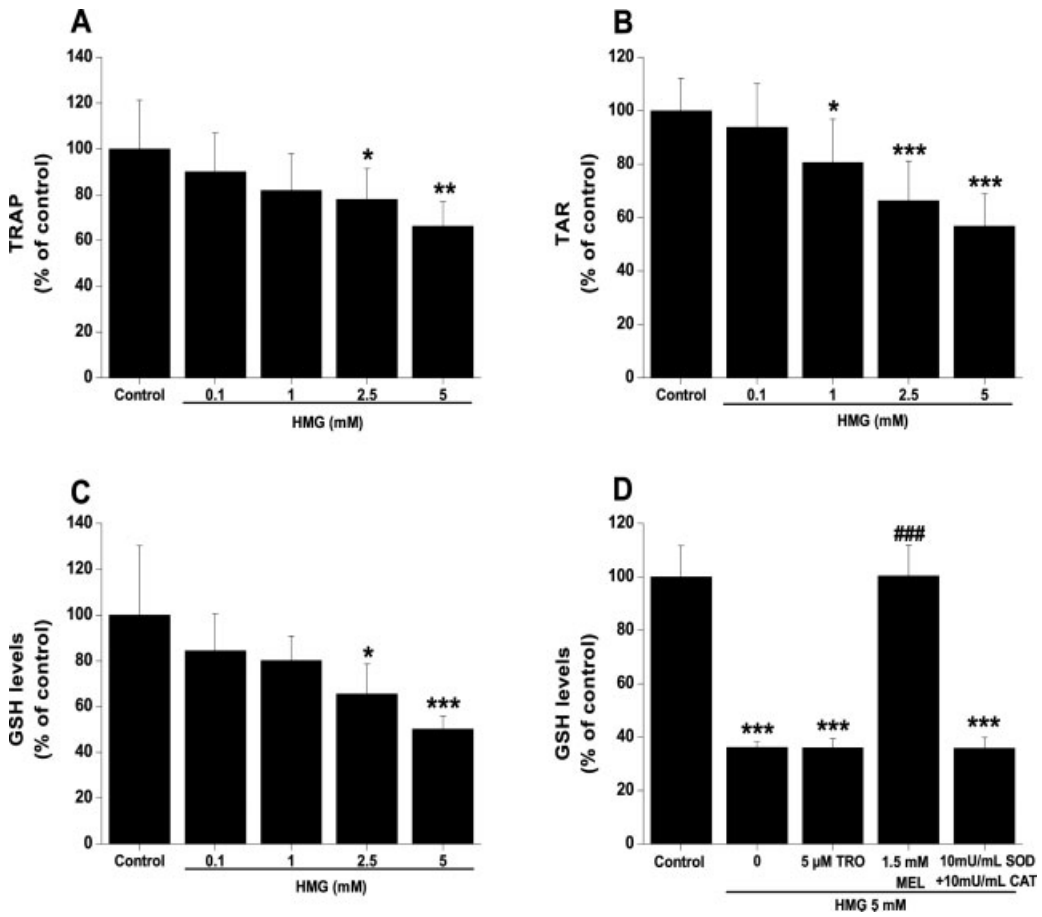


Fig. 5. **A–D:** In vitro effect of 3-hydroxy-3-methylglutarate (HMG) on the nonenzymatic antioxidant defenses total-radical trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), and glutathione (GSH) levels in rat cerebral cortex supernatants. In some experiments, cortical supernatants were preincubated for 15 min with the antioxidants Trolox (TRO; 5 μM), melatonin (MEL; 1,500 μM) or the combination of catalase (CAT) plus superoxide dismutase (SOD; 10 mU/ml of each enzyme) before the addition of 5.0 mM HMG and GSH levels measured afterward. Values are means ± standard deviation for five to seven independent experiments performed in triplicate and are expressed as percentage of controls [controls: (A) TRAP values: 12.8 ± 4.5 nmol/mg protein; (B) TAR values: 12.9 ± 2.47 nmol/mg of protein; (C) GSH levels: 24.1 ± 7.25 nmol/mg protein; (D) GSH levels: 9.59 ± 1.12 nmol/mg protein]. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$ compared with controls; $^{\#\#\#}P < 0.001$, compared with 5.0 mM HMG (Duncan multiple range test).

TABLE I. Effect of 3-Hydroxy-3-Methylglutarate (HMG) on Commercial Reduced Glutathione (GSH) and on Purified Mitochondrial Membrane Protein-Bound Sulfhydryl Groups (PBSG) From Rat Cortical Supernatants[†]

GSH oxidation	
Control	100 ± 4.04
5.0 mM HMG	107 ± 8.94
250 μM NEM	71.5 ± 31***
Mitochondrial PBSG oxidation	
Control	100 ± 21.6
5.0 mM HMG	98.8 ± 6.4
250 μM PHMB	62 ± 29***

[†]PHMB, *p*-hydroxymercuribenzoic acid; NEM, *N*-ethylmaleimide (positive controls). Values are means ± standard deviation for three to five independent experiments performed in triplicate and are expressed as percentage of controls. GSH (200 μM) oxidation was measured in the absence of brain tissue. Controls: GSH oxidation: 1,052 ± 42 fluorescence units; mitochondrial PBSG oxidation: 74.0 ± 16 nmol/mg protein.
$^{***}P < 0.001$ compared with controls (Duncan multiple-range test).

Hähnel, 1986; Thompson et al., 1990; Sweetman et al., 2001). Although the neurological symptoms are common and cerebral MRI reveals marked lesions in periventricular and/or subcortical white matter and arcuate fibers, the mechanisms underlying the brain damage in this disorder are not yet established.

In the present investigation, we have demonstrated that HMG, at concentrations as low as 0.25 mM, significantly increased chemiluminescence and TBA-RS levels in cerebral cortical supernatants. Light in the chemiluminescence assay arises mainly from excited carbonyls, O_2.-, $ONOO^-$, and from peroxidizing lipids (Halliwell and Gutteridge, 2007a,b). On the other hand, TBA-RS measurement reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge, 2007a,b). Therefore, the increased values of these parameters elicited by relatively low concentrations of HMG strongly indicate

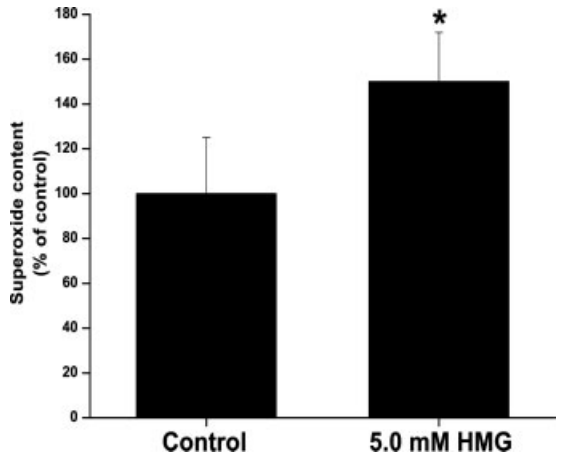


Fig. 6. In vitro effect of 3-hydroxy-3-methylglutarate (HMG) on superoxide generation in submitochondrial particles from rat cerebral cortex. Values are means ± standard deviation for five independent experiments performed in triplicate and are expressed as percentage of controls (control: 11.2 ± 2.77 nmol/min · mg protein). $^{\star}P < 0.05$ compared with control (Student's *t*-test for paired samples).

TABLE II. *In Vitro* Effect of 3-Hydroxy-3-Methylglutarate (HMG) on the Activities of the Antioxidant Enzymes Catalase (CAT), Glutathione Peroxidase (GPx), and Superoxide Dismutase (SOD) in Rat Cerebral Cortex Supernatants⋆

	HMG (mM)		
Enzyme activities	0	2.5	5.0
CAT (n = 4)	100 ± 11	104 ± 11	104 ± 14
GPx (n = 4)	100 ± 14	103 ± 12	100 ± 9
SOD (n = 3)	100 ± 18	104 ± 22	101 ± 11

⋆Cerebral cortex supernatants were incubated during 60 min with 2.5 or 5.0 mM HMG and had their activities measured afterward. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as percentage of controls. One unit is defined as 1 μmol H_2O_2 consumed per minute for CAT, 1 μmol NADPH consumed per minute for GPx, and 50% of produced chromogen inhibition for SOD. Controls: CAT activity: 2.87 ± 0.31 U/mg protein; GPx activity: 20.45 ± 2.77 U/mg protein; SOD activity: 4.82 ± 0.86 U/mg protein (one-way ANOVA).

that HMG caused lipid peroxidation in vitro in cerebral cortex. We also observed that the lipid oxidative damage induced by HMG was totally prevented by high doses of the free radical scavengers TRO (α-tocopherol), MEL, and the combination of SOD plus CAT, although smaller doses of TRO or SOD plus CAT, as well as GSH, L-NAME and Cr were not able to change the HMG-induced increase on TBA-RS values. Furthermore, relatively low concentrations of MEL significantly attenuated and NAC fully prevented the increase of TBA-RS measurement provoked by HMG. These data indicate that HMG provoked a strong prooxidant effect on membrane lipids from cerebral cortex probably mediated by reactive oxygen species generation. We also found that HMG did not change TBA-RS levels in intact and disrupted mitochondrial preparations (postmitochondrial supernatants), suggesting that the generation of oxidants by this organic acid causing lipid damage occurred via cytosolic rather than mitochondrial mechanisms.

Protein carbonyl formation (PCF) and sulfhydryl oxidation were also induced by a wide range of HMG concentrations (0.5 mM–5.0 mM) in cortical supernatants, indicating that this organic acid also provokes protein oxidative damage. Considering that HMG was not able directly to oxidize thiol groups from commercial (GSH) and brain purified mitochondrial preparations, it is presumed that HMG induced protein oxidative damage via reactive species generation (Reznick and Packer, 1993; Levine, 2002).

With regard to the antioxidant defense system, HMG markedly reduced the total content of antioxidants (TRAP) and of GSH, the main naturally occurring antioxidant, as well as the capacity to handle increased levels of reactive species (TAR). Because these parameters are used to evaluate the nonenzymatic antioxidant capacity of a tissue to prevent the damage associated with free radical processes, it can be concluded that the rat cortical nonenzymatic antioxidant defenses were severely compromised by HMG (Lissi et al., 1995; Evelson et al., 2001; Halliwell and Gutteridge, 2007a,b). We also verified that MEL, but not TRO or SOD plus CAT, was able to prevent the HMG decrease of reduced glutathione (GSH) levels in cortical supernatants, suggesting that the highly toxic hydroxyl radical, which is the principal species scavenged by MEL (Reiter, 1998), was probably involved in the reduction of GSH provoked by HMG. These results along with those showing that MEL, TRO, and SOD plus CAT fully prevented the in vitro stimulation of lipid peroxidation elicited by HMG suggest that the free radicals hydroxyl, peroxyl, and superoxide, which are scavenged by these antioxidants (Reiter et al., 1997a,b, 2001; Anisimov et al., 2006), are involved in the prooxidant effects of HMG. Furthermore, our experiments showing that superoxide generation is activated by HMG in cortical mitochondria and the significant inverse correlation found between TBA-RS levels and GSH concentrations reinforce the view that the decrease of the brain antioxidant defenses and the induction of oxidative damage caused by HMG were mediated by reactive species.

We cannot at present establish the exact signal transduction cascades by which HMG induced lipid peroxidation and protein oxidative damage in cortical supernatants. However, it may be presumed that the free radicals elicited by this organic acid could initiate the classical cascades leading to lipid and protein oxidation (carbonyl formation and sulfhydryl oxidation; Halliwell and Gutteridge, 2007b).

We also verified that the activities of the antioxidant enzymes CAT, GPx, and SOD were not altered by exposing cortical supernatants for 60 min to HMG. However, we cannot exclude the possibility that sustained high concentrations of HMG (in vivo studies)

could lead to up-regulation of these enzymes via gene transcription in order to counterbalance free radical generation, as occurs in other pathological conditions involving oxidative stress (Karelson et al., 2001; Genet et al., 2002; Jafari, 2007).

Insofar as oxidative stress results from an imbalance between the total antioxidant defenses and the reactive species generated in a tissue, the present data strongly indicate that HMG induces oxidative stress in rat cerebral cortex, a deleterious cell condition (Halliwell and Gutteridge, 2007a,b). At this point, it should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues (Halliwell and Gutteridge, 1996), a fact that makes this tissue more vulnerable to increased reactive species. In fact, oxidative stress has been implicated in the pathophysiology of common neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, as well as in epileptic seizures and demyelination (Reznick and Packer, 1993; Halliwell and Gutteridge, 1996; Perez-Severiano et al., 2000; Bogdanov et al., 2001; Méndez-Álvarez et al., 2001; Karelson et al., 2001; Behl and Moosmann, 2002; Stoy et al., 2005; Berg and Youdim, 2006; Mancuso et al., 2006).

On the other hand, it is difficult to determine the pathophysiological relevance of our data, because to our knowledge brain concentrations of HMG in HMGLD affected patients are not yet established. However, it should be noted that the significant alterations in the oxidative stress parameters evaluated occurred with relatively low doses of HMG (0.25 mM and higher), and severe neurological symptoms in these patients occur especially during metabolic crises, in which the concentrations of the accumulating metabolites dramatically increase. Furthermore, it has been proposed that the concentrations of organic acids, particularly dicarboxylic acids (including 3-hydroxy-3-methylglutarate), that accumulate in various organic acidemias are higher in the brain compared with the serum because these compounds are trapped in neural cells (Hoffmann et al., 1993, 1994; Sauer et al., 2006; Stellmer et al., 2007).

In conclusion, the present data indicate that oxidative stress is induced by HMG in brain of young rats. If the present in vitro findings are confirmed in vivo in animal experiments and also in tissues from patients affected by HMGLD, it would be tempting to speculate that reactive species may contribute, at least in part, to the neurological damage found in this disorder. Finally, it may be proposed that the administration of antioxidants should be considered as an adjuvant therapy to specific diets or other pharmacological agents for these patients, especially during metabolic crises.

REFERENCES

Aebi H. 1984. Catalase, in vitro. Methods Enzymol 105:121–126.

Aksenov MY, Markesbery WR. 2001. Change in thiol content and expression of glutathione redox system gene in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 302:141–145.

Anisimov VN, Popovich IG, Zabezhinski MA, Anisimov SV, Vesnushkin GM, Vinogradova IA. 2006. Melatonin as antioxidant, geroprotector and anticarcinogen. Biochim Biophys Acta 1757:573–589.

Behl C, Moosmann B. 2002. Oxidative nerve cell death in Alzheimer's disease and stroke: antioxidants as neuroprotective compounds. Biol Chem 383:521–536.

Berg D, Youdim MB. 2006. Role of iron in neurodegenerative disorders. Top Magn Reson Imaging 17:5–17.

Bogdanov MB, Andreassen OA, Dedeoglu A, Ferrante RJ, Beal MF. 2001. Increased oxidative damage to DNA in a transgenic mouse of Huntington's disease. J Neurochem 79:1246–1249.

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.

Browne RW, Armstrong D. 1998. Reduced glutathione and glutathione disulfide. Methods Mol Biol 108:347–352.

Esterbauer H, Cheeseman KH. 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol 186:407–421.

Evelson P, Travacio M, Repetto M, Escobar J, Llesuy S, Lissi E. 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch Biochem Biophys 388:261–266.

Faull KF, Bolton PD, Halpern B, Hammond J, Dankes DM. 1976. The urinary organic acid profile associated with 3-hydroxy-3-methylglutaric aciduria. Clin Chim Acta 73:553–559.

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 73:268–275.

Genet S, Kale RK, Baquer NZ. 2002. Alterations in antioxidant enzymes and oxidative damage in experimental diabetic rat tissues: Effect of vanadate and fenugreek (*Trigonella foenum graecum*). Mol Cell Biochem 236:7–12.

Gibson KM, Breuer J, Kaiser K, Nyhan WL, McCoy EE, Ferreira P, Greene CL, Blitzer MG, Shapira E, Reverte F, Conde C, Bagnell P, Cole DEC. 1988a. 3-Hydroxy-3-methylglutarylcoenzyme A lyase deficiency: report of five new patients. J Inherit Metab Dis 11:76–87.

Gibson KM, Breuer J, Nyhan WL. 1988b. 3-Hydroxy-3-methylglutarylcoenzyme A lyase deficiency: review of 18 reported patients. Eur J Pediatr 148:180–186.

Gibson KM, Cassidy SB, Seaver LH, Wanders RJ, Kennaway NG, Mitchell GA, Spark RP. 1994. Fatal cardiomyopathy associated with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. J Inherit Metab Dis 17:291–304.

Gonzalez-Flecha B, Llesuy S, Boveris A. 1991. Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver, and muscle. Free Radic Biol Med 10:93–100.

Halliwell B, Gutteridge JMC. 1996. Oxygen radicals and nervous system. Trends Neurosci 8:22–26.

Halliwell B, Gutteridge JMC. 2007a. Measurement of reactive species. In: Halliwell B, Gutteridge JMC, editors. Free radicals in biology and medicine. Oxford: Oxford University Press. p 268–340.

Halliwell B, Gutteridge JMC. 2007b. Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. In: Halliwell B, Gutteridge JMC, editors. Free radicals in biology and medicine. Oxford: Oxford University Press. p 187–267.

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.

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.

Jafari M. 2007. Dose- and time-dependent effects of sulfur mustard on antioxidant system in liver and brain of rat. Toxicology 231:30–39.

Karelson E, Bogdanovic N, Garlind A, Winblad B, Zilmer K, Kullisaar T, Vihalemm T, Kairane C, Zilmer M. 2001. The cerebrocortical areas in normal brain aging and in the Alzheimer's disease: noticeable difference in the lipid peroxidation level and in antioxidant defense. Neurochem Res 26:353–361.

Kowaltowski AJ, Vercesi AE, Castilho RF. 1997. Mitochondrial membrane protein thiol reactivity with N-ethylmaleimide or mersalyl is modified by Ca2+: correlation with mitochondrial permeability transition. Biochim Biophys Acta 1318:395–402.

Lee C, Tsai FJ, Wu JY, Peng CT, Tsai CH, Hwu WL, Wang TR, Millington DS. 1999. 3-Hydroxy-3-methylglutaric aciduria presenting with Reye like syndrome: report of one case. Acta Paediatr Taiwan 40:445–447.

Levine RL. 2002. Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radic Biol Med 32:790–796.

Lissi E, Pascual C, Del Castillo MD. 1992. Luminol luminescence induced by 2,2-azo-bis-(2-amidinopropane) thermolysis. Free Radic Res Commun 17:299–311.

Lissi E, Salim-Hanna M, Pascual C, Del Castillo MD. 1995. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. Free Radic Biol Med 18:153–158.

Lowry OH, Rosebrough NJ, Lewis-Farr A, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Lyon G, Adams RD, Kolodny EH. 1996. The neurology of neonatal hereditary metabolic diseases. In: Lyon G, Adams RD, Kolodny EH, editors. Neurology of hereditary metabolic disease of children. New York: McGraw Hill. p 29–30.

Mancuso M, Coppede F, Migliore L, Siciliano G, Murri L. 2006. Mitochondrial dysfunction, oxidative stress and neurodegeneration. J Alzheimer Dis 10:59–73.

Marklund SL. 1985. Pyrogallol autoxidation. In: Handbook for oxygen radical researh. Boca Raton, FL: CRC Press. p 243–247.

Méndez-Álvarez E, Soto-Otero R, Hermida-Aeijeiras A, López-Real AM, Labandeira-García JL. 2001. Effects of aluminium and zinc on the oxidative stress caused by 6-hydroxydopamine autoxidation: relevance for the pathogenesis of Parkinson's disease. Biochim Biophys Acta 1586:155–168.

Perez-Severiano F, Rios C, Segovia J. 2000. Striatal oxidative damage parallels the expression of a neurological phenotype in mice transgenic for the mutation of Huntington's disease. Brain Res 862:234–237.

Poderoso JJ, Carreras MC, Lisdero C, Riobó N, Schöpfer F, Boveris A. 1996. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. Arch Biochem Biophys 328:85–92.

Reiter RJ. 1998. Oxidative damage in the central nervous system: protection by melatonin. Prog Neurobiol 56:359–384.

Reiter RJ, Carneiro RC, Oh CS. 1997a. Melatonin in relation to cellular antioxidative defense mechanisms. Horm Metab Res 29:363–372.

Reiter R, Tang L, Garcia JJ, Munoz-Hoyos A. 1997b. Pharmacological actions of melatonin in oxygen radical pathophysiology. Life Sci 60:2255–2271.

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 34:237–256.

Reznick AZ, Packer L. 1993. Free radicals and antioxidants in muscular neurological diseases and disorders. In: Poli G, Albano E, Dianzani UM, editors. Free radicals: from basic science to medicine. Basel: Birkhauser Verlag. p 425–437.

Reznick AZ, Packer L. 1994. Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. Methods Enzymol 233:357–363.

Sauer SW, Okun JG, Fricker G, Mahringer A, Müller I, Crnic LS, 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 constitute a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. J Neurochem 97:899–910.

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, Muhlhausen C. 2007. 3-Hydroxyglutaric acid is transported via the sodium-dependent dicarboxylate transporter NaDC3. J Mol Med (in press).

Stoy N, Mackay GM, Forrest CM, Christofides J, Egerton M, Stone TW, Darlington LG. 2005. Tryptophan metabolism and oxidative stress in patients with Huntington's disease. J Neurochem 93:611–623.

Sweetman L, Williams JC. 2001. Branched chain organic acidurias. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill. p 2340–2342.

Thompson GN, Chalmers RA, Halliday D. 1990. The contribution of protein catabolism to metabolic decompensation in 3-hydroxy-3-methylglutaric aciduria. Eur J Pediatr 149:346–350.

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.

Wendel A. 1981. Glutathione peroxidase. Methods Enzymol l77:325–332.

Wysocki SJ, Hähnel R. 1986. 3-Hydroxy-3-methylglutaryl-coenzyme a lyase deficiency: a review. J Inherit Metab Dis 9:225–233.

Wysocki SJ, Wilkonson SP, Haehnel R, Wong CYB, Panegyres PK. 1976. 3-Hydroxy-3-methylglutaric aciduria, combined with 3-methylglutaconic aciduria. Clin Chim Acta 70:399–406.

Yalcinkaya C, Dincer A, Gündüz E, Ficicioglu C, Kocer N, Aydin A. 1999. MRI and MRS in HMG-CoA lyase deficiency. Pediatr Neurol 20:375–380.

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

Capítulo II

Induction of oxidative stress by the metabolites accumulating in 3-methylglutaconic aciduria in cerebral cortex of young rats

Guilhian Leipnitz, Bianca Seminotti, Alexandre U. Amaral, Giorgia de M. de Bortoli, Alexandre Solano, Patrícia F. Schuck, Ângela T. S. Wyse, Clóvis M. D. Wannmacher, Alexandra Latini, Moacir Wajner.

Artigo científico publicado no periódico

Life Sciences

Available online at www.sciencedirect.com
ScienceDirect
Life Sciences 82 (2008) 652–662
Life Sciences
www.elsevier.com/locate/lifescie

Induction of oxidative stress by the metabolites accumulating in 3-methylglutaconic aciduria in cerebral cortex of young rats

Guilhian Leipnitz [a], Bianca Seminotti [a], Alexandre U. Amaral [a], Giorgia de Bortoli [a], Alexandre Solano [a], Patrícia F. Schuck [a], Ângela T.S. Wyse [a], Clóvis M.D. Wannmacher [a], Alexandra Latini [c], Moacir Wajner [a,b,d,*]

[a] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul, Porto Alegre — RS, Brazil*
[b] *Hospital de Clínicas, Serviço de Genética Médica, Porto Alegre — RS, Brazil*
[c] *Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis — SC, Brazil*
[d] *Universidade Luterana do Brasil, Canoas — Brazil*

Received 8 October 2007; accepted 18 December 2007

Abstract

3-Methylglutaconic (MGT), 3-methylglutaric (MGA) and occasionally 3-hydroxyisovaleric (OHIVA) acids accumulate in a group of diseases known as 3-methylglutaconic aciduria (MGTA). Although the clinical presentation of MGTA is mainly characterized by neurological symptoms, the mechanisms of brain damage in this disease are poorly known. In the present study we investigated the in vitro effect of MGT, MGA and OHIVA on various parameters of oxidative stress in cerebral cortex from young rats. Thiobarbituric acid-reactive substances (TBA-RS) and chemiluminescence were significantly increased by MGT, MGA and OHIVA, indicating that these metabolites induce lipid oxidative damage. Furthermore, the addition of melatonin, α-tocopherol and superoxide dismutase plus catalase fully prevented MGT-induced increase on TBA-RS, suggesting that free radicals were involved in this effect. These metabolites also provoked protein oxidative damage determined by increased carbonyl formation and sulfhydryl oxidation, but did not induce superoxide generation in submitochondrial particles. It was also verified that MGA and MGT significantly decreased the non-enzymatic antioxidant defenses in cerebral cortex supernatants and that melatonin and α-tocopherol totally blocked MGA-induced GSH reduction. The data indicate that the metabolites accumulating in MGTA elicit oxidative stress in vitro in the cerebral cortex. It is therefore presumed that this pathomechanism may be involved in the brain damage observed in patients affected by MGTA.

Keywords: 3-Methylglutaconic aciduria; Oxidative stress; Cerebral cortex

Introduction

3-Methylglutaconic aciduria (MGTA) comprehends a group of metabolic disorders characterized by increased urinary excretion of 3-methylglutaconic acid (MGT) and 3-methylglutaric acid (MGA). So far, four distinct forms of MGTA have been described. MGTA caused by 3-methylglutaconyl-CoA hydratase (EC 4.2.4.18) deficiency (MGTA type I) is an inborn error of leucine catabolism (McKusick 250950) and differs from the other types by the simultaneous urinary excretion of increased amounts of 3-hydroxyisovaleric acid (OHIVA). Clinical features of MGTA type I are nonspecific and variable, ranging from minimal to severe symptoms, including delayed speech and language development with hypoglycemia and metabolic acidosis (Sweetman and Williams, 2001). Psychomotor retardation, microcephaly and progressive neurological impairment with spastic quadriplegia, seizures, and dystonia have also been reported in several patients (Shoji et al., 1999; Ijlst et al., 2002; Arn and Funanage, 2006; Di Rosa et al., 2006).

* Corresponding author. 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 — Brazil. Tel.: +55 51 3308 5571; fax: +55 51 3308 5535.
E-mail address: mwajner@ufrgs.br (M. Wajner).

0024-3205/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2007.12.024

Furthermore, tissues with higher requirement for oxidative metabolism, such as the central nervous system, cardiac and skeletal muscle, are predominantly affected (Gunay-Aygun, 2005). Patients with MGTA type I excrete higher levels of MGT and MGA than those affected by other variants of MGTA.

Barth syndrome, or MGTA type II (McKusick 302060), is an X-linked disorder characterized by dilated cardiomyopathy, neutropenia and skeletal myopathy (Barth et al., 2004), possibly related to an impairment in cardiolipin metabolism due to mutations in the tafazzin gene (Bione et al., 1996). MGTA type III, also known as Costeff optic atrophy syndrome (McKusick 258501), is an early-onset, autossomal recessive syndrome of infantile bilateral optic atrophy and neurologic defects caused by a mutation in the *OPA3* gene (Anikster et al., 2001; Arn and Funanage, 2006). Besides these well-defined forms of MGTA, an unclassified group known as type IV has been reported in many patients presenting variable psychomotor retardation, spasticity, hypertonicity and cardiomyopathy (Gibson et al., 1991, 1993; Gunay-Aygun, 2005). Some MGTA patients have elevated lactic acid or citric acid cycle intermediates, as well as abnormalities of the mitochondrial electron transport chain (Ibel et al., 1993; Besley et al., 1995; Ruesch et al., 1996), including deficiency of the activities of respiratory chain complexes I, II, III, IV and V (Gibson et al., 1993; Sweetman and Williams, 2001; Gunay-Aygun, 2005; Wortmann et al., 2006), strongly indicating mitochondrial dysfunction.

Although neurological symptoms are common in MGTA, the underlying mechanisms of brain damage in this disorder are not yet established. To our knowledge, the only available data on the effects of the metabolites accumulating in this disorder evidenced that OHIVA does not alter brain bioenergetics (Ribeiro et al., 2007), whereas the other acidic compounds were not tested. Therefore, in the present work we investigated the in vitro effect of MGT, MGA and OHIVA, the major metabolites accumulating in MGTA, on various parameters of oxidative stress, namely thiobarbituric acid-reactive substances (TBA-RS), chemiluminescence, carbonyl formation, oxidation of sulfhydryl (thiol) groups, superoxide formation, total radical-trapping antioxidant potential (TRAP) and glutathione (GSH) levels in cerebral cortex of young rats. The role of antioxidants on some effects elicited by these compounds was also studied.

Materials and methods

Animals and reagents

Wistar rats of 30 days of life 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 °C ± 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 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.

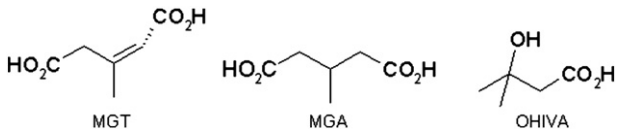


Fig. 1. Chemical structures of 3-methylglutaconic aciduria (MGTA)-related compounds: 3-methylglutaconic acid (MGT), 3-methylglutaric acid (MGA) and 3-hydroxyisovaleric acid (OHIVA).

All chemicals were purchased from Sigma (St. Louis, MO, USA), except for MGT and OHIVA which were prepared by Dr. Ernesto Brunet. MGT, MGA and OHIVA solutions were prepared on the day of the experiments in the incubation medium used for each technique and pH was adjusted at 7.4. The final concentrations of the acids in the medium ranged from 0.1 to 5.0 mM. Fig. 1 shows the chemical structures of the major metabolites accumulating in MGTA. In some experiments, antioxidants were added to the incubation medium at the following final concentrations: 1.5–10 μM Trolox (TRO), 100 μM GSH, 500 μM N^{ω}-nitro-L-arginine (L-NAME), 200–1000 μM melatonin (MEL) and the combination of SOD plus CAT (2.5–15 mU/mL each). Chemiluminescence and TRAP were assayed using a Wallac 1409 scintillation counter. TBA-RS, carbonyl and the antioxidant enzyme activities were measured with a double-beam Hitachi U-2001 spectrophotometer with temperature control. GSH levels were measured in a Hitachi F-2000 fluorescence spectrophotometer.

Cerebral cortex supernatant preparation and incubation

On the day of the experiments the rats were sacrificed by decapitation without anaesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum and striatum were discarded, and the cerebral cortex was dissected, weighed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated at 37 °C for 1 h with MGT, MGA or OHIVA at concentrations of 0.1, 1, 2.5 and 5 mM. Controls did not contain the metabolites in the incubation medium. Immediately after incubation, aliquots were taken to measure TBA-RS, chemiluminescence, TRAP, GSH concentrations, carbonyl content and sulfhydryl oxidation.

Mitochondrial membrane preparation

Cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 10 mM Tris, pH 7.4 containing 0.25 M sucrose and the homogenates centrifuged at 650 *g* for 10 min to discard nuclei and cell debris (Kowaltowski et al., 1997). After a new centrifugation at 25,000 *g* for 15 min, the resulting pellet containing

the mitochondria was suspended in 0.1 M Tris, pH 8.0 and stored in aliquots at −70 °C. On the day of the experiment, the aliquots of purified mitochondria were frozen/thawed three times and centrifuged at 15,000 *g* for 2 min to separate the mitochondrial membranes. The resulting pellet was washed with 6.5% TCA three times and centrifuged at 15,000 *g* for 2 min. The final pellet was suspended in 0.5 M Tris buffer, pH 8.3, containing 0.5 mM EDTA and incubated at 37 °C for 1 h with 5 mM MGT, MGA and OHIVA to measure the mitochondrial membrane sulfhydryl (thiol)-bound content.

Isolation of submitochondrial particles

Submitochondrial particles were prepared at 4 °C from frozen and thawed mitochondria (20 mg protein/mL) according to Poderoso et al. (1996). The obtained submitochondrial particles were washed twice with 140 mM KCl, 20 mM Tris–HCl, pH 7.4, and suspended in the same medium. The suspended particles were then incubated at 37 °C for 1 h with 5 mM MGT, MGA and OHIVA. Immediately after incubation, aliquots from this preparation were used to measure superoxide formation.

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid was added to 150 μL of pre-treated cerebral cortex supernatants and centrifuged at 300 *g* for 10 min. Three hundred μL of the supernatants was transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulphate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-

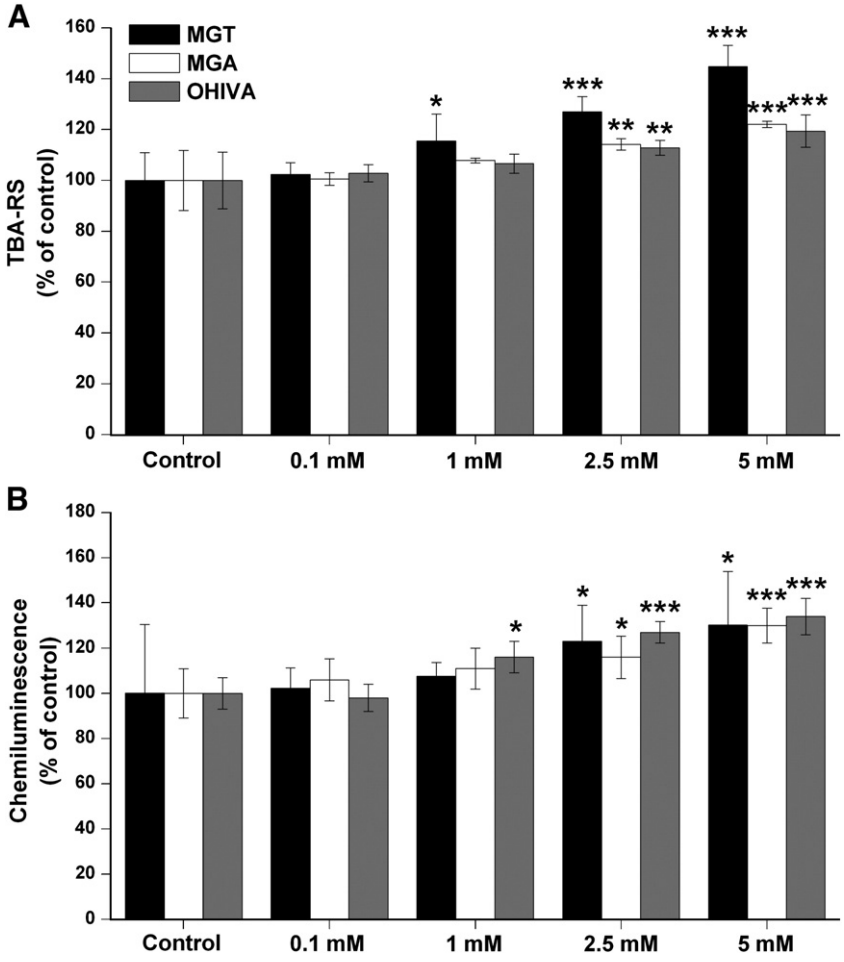


Fig. 2. In vitro effect of 3-methylglutaconate (MGT), 3-methylglutarate (MGA) and 3-hydroxyisovalerate (OHIVA) on the lipid peroxidation parameters thiobarbituric acid-reactive substances (TBA-RS) (A) and chemiluminescence (B) in rat cerebral cortex supernatants. Cortical supernatants were incubated during 60 min in the presence of MGT, MGA and OHIVA at concentrations ranging from 0.1 to 5.0 mM. Values are means±standard deviation for five to six independent experiments performed in triplicate and are expressed as percentage of controls (Controls TBA-RS levels (A): MGT: 3.05±0.33 nmol/mg protein; MGA: 3.98±0.58 nmol/mg protein; OHIVA: 4.16±0.46 nmol/mg protein; Chemiluminescence (B): MGT: 3447±693 cpm/mg protein; MGA: 1644±180 cpm/mg protein; OHIVA: 1992±292 cpm/mg protein). *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared to controls (Duncan multiple range test).

RS values were calculated as nmol/mg protein and represented as percentage of control.

Chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room by the method of Gonzalez-Flecha et al. (1991). Incubation flasks contained 3.5 mL of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl were counted for background chemiluminescence during 5 min. An aliquot of 500 μL of cortical supernatants was immediately added and chemiluminescence was measured for 30 min at room temperature. The background chemiluminescence was subtracted from the final luminescence value and the results were calculated as cpm/mg protein and represented as percentage of control.

Determination of protein carbonyl content

PCF (protein carbonyl formation), a marker of protein oxidative damage, was measured spectrophotometrically

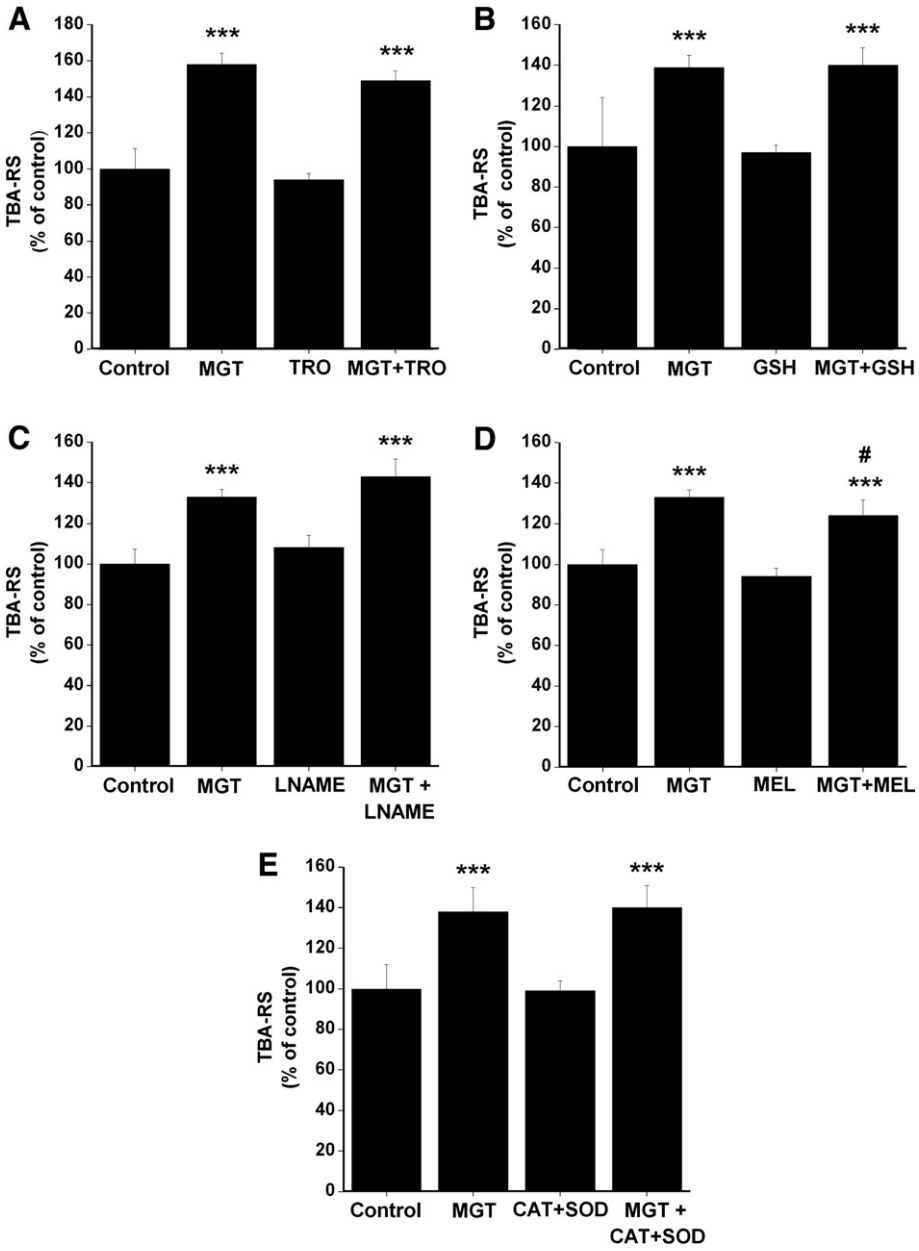


Fig. 3. Effect of antioxidants on 3-methylglutaconate (MGT)-induced in vitro lipid peroxidation (TBA-RS) in rat cerebral cortex supernatants. Cortical supernatants were pre-incubated for 15 min with 1.5 μM α-tocopherol (Trolox) (A), 100 μM glutathione (GSH) (B), 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (C), 200 μM melatonin (MEL) (D) or the combination of 2.5 mU/mL catalase (CAT) plus 2.5 mU/mL superoxide dismutase (SOD) (E) before the addition of 5.0 mM MGT. Values are means ± standard deviation for four to five independent experiments performed in triplicate and are expressed as percentage of controls (Controls [nmol/mg protein]: A: 4.64 ± 0.52; B: 4.39 ± 1.04; C: 2.11 ± 0.15; D: 2.11 ± 0.15; E: 4.99 ± 0.6). ***$P<0.001$, compared to controls; $^{\#}P<0.05$, compared to 5.0 mM MGT (Duncan multiple range test).

according to Reznick and Packer (1994). One hundred microliters of the aliquots from the incubation was treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine (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 500 μL 20% TCA and centrifuged for 5 min at 10,000 *g*. The pellet was then washed with 1mL ethanol:ethyl acetate (1:1, V/V) and dissolved in 550 μL 6 M guanidine prepared in 2.5 N HCl at 37 °C for 5 min. 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 and represented as percentage of control, using the extinction coefficient of $22{,}000 \times 10^6$ nmol/mL for aliphatic hydrazones.

Total radical-trapping antioxidant potential (TRAP)

TRAP, representing the total non-enzymatic antioxidant capacity of the tissue, was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. (1992). The reaction mixture containing 4 mL 10 mM ABAP dissolved in 0.1 M glycine buffer, pH 8.6 and 10 μL of luminol (4 mM) generates, at room temperature, an almost constant light intensity corresponding to free radical formation, which was measured in a Wallac 1409 liquid scintillation counter. This was considered to be the initial chemiluminescence values. Then, 10 μL of 300 μM trolox (soluble α-tocopherol analogue) or 50 μL of cortical supernatants was added to the reaction medium. The addition of trolox or supernatants provokes a marked reduction of the light intensity, which is maintained for a certain period after which light intensity rapidly increase. This period corresponds to induction time (IT) and represents TRAP measurement. IT is directly proportional to the antioxidant capacity of the tissue, and the IT of each sample was compared with the IT of trolox. TRAP values were calculated as nmol trolox/mg of protein and expressed as percentage of control.

Reduced glutathione (GSH) content

GSH concentrations were measured according to Browne and Armstrong (1998). Aliquots from the incubation were diluted in 20 volumes of (1:20, v/v) 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred μL of this preparation was incubated with an equal volume of *o*-phthaldialdehyde (1 mg/mL methanol) at room temperature during 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.01–1 mM) and the concentrations were calculated as nmol/mg protein and represented as percentage of control.

Sulfhydryl content

This assay is based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Kowaltowski et al., 1997). *p*-Hydroxymercuribenzoic acid (250 μM) was used as a positive control. Briefly, 40 μL of mitochondrial membrane

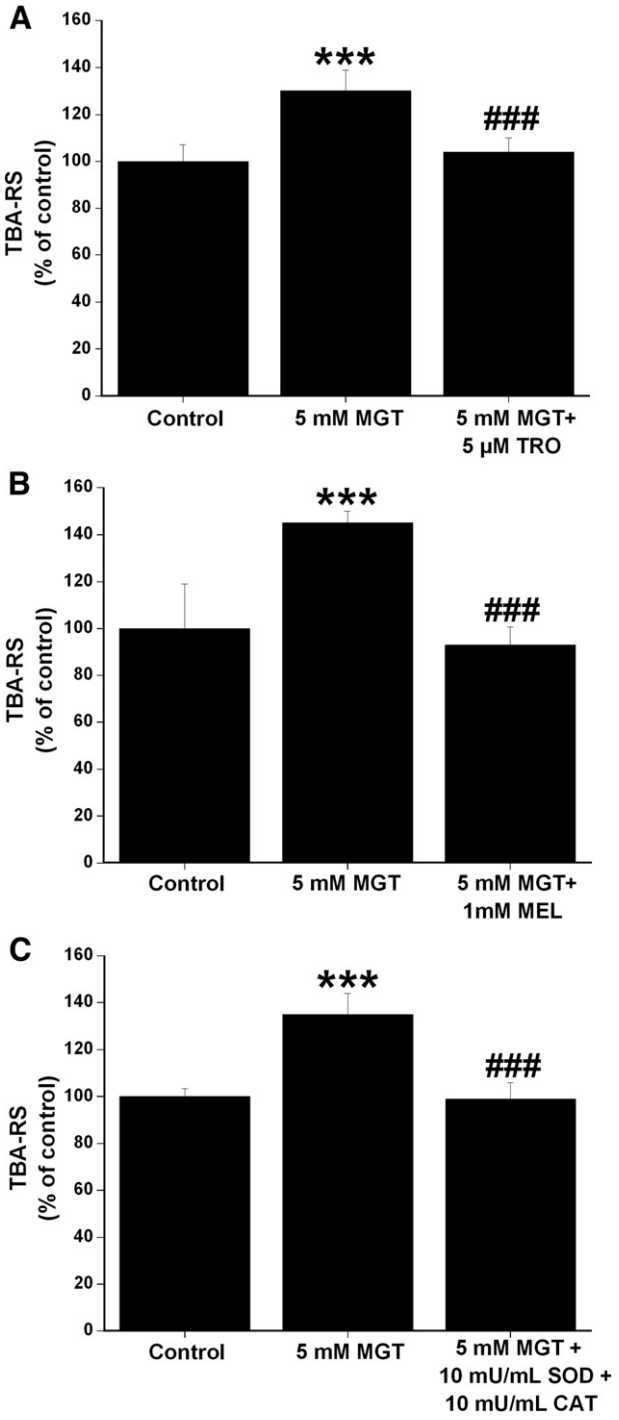


Fig. 4. Effect of increasing concentrations of antioxidants on 3-methylglutaconate (MGT)-induced in vitro lipid peroxidation (TBA-RS) in rat cerebral cortex supernatants. Cortical supernatants were pre-incubated for 15 min with the antioxidants Trolox (TRO, 5 μM) (A), melatonin (MEL; 1000 μM) (B) or the combination of catalase (CAT) plus superoxide dismutase (SOD) (10 mU/mL of each enzyme) (C) before the addition of 5.0 mM MGT. Values are means±standard deviation of five independent experiments performed in triplicate and are expressed as percentage of controls (Controls [nmol/mg protein]: TRO: 5.31±0.38; MEL: 4.29±0.23; SOD+CAT: 3.96±0.38). ***$P<0.001$, compared to controls; ###$P<0.01$, compared to 5.0 mM MGT (Duncan multiple range test).

suspension or 120 μL of cortical supernatant was incubated at 37 °C for 1 h with MGT, MGA and OHIVA at concentrations of 0.1, 1, 2.5 and 5 mM. Then, DTNB was added. This was followed by a 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein and represented as percentage of control.

Superoxide content

Superoxide production was determined spectrophotometrically according to Poderoso et al. (1996) after exposition of submitochondrial particles to MGT, MGA or OHIVA. The assay is based on superoxide-dependent oxidation of epinephrine to adenochrome at 37 °C ($E_{480\ nm}$=4.0 mM/cm). The reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, pH 7.4, 0.1 mM catalase, 1 mM epinephrine and 7 mM succinate. Superoxide dismutase was used at 0.1–0.3 mM final concentrations as a negative control to confirm assay specificity. Results were calculated as nmol/min mg protein and represented as percentage of control.

Protein determination

Protein content was determined in cerebral cortex supernatants by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F value was significant. Linear regression analysis was also used to test dose-dependent effects. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $P<0.05$ was considered to be significant.

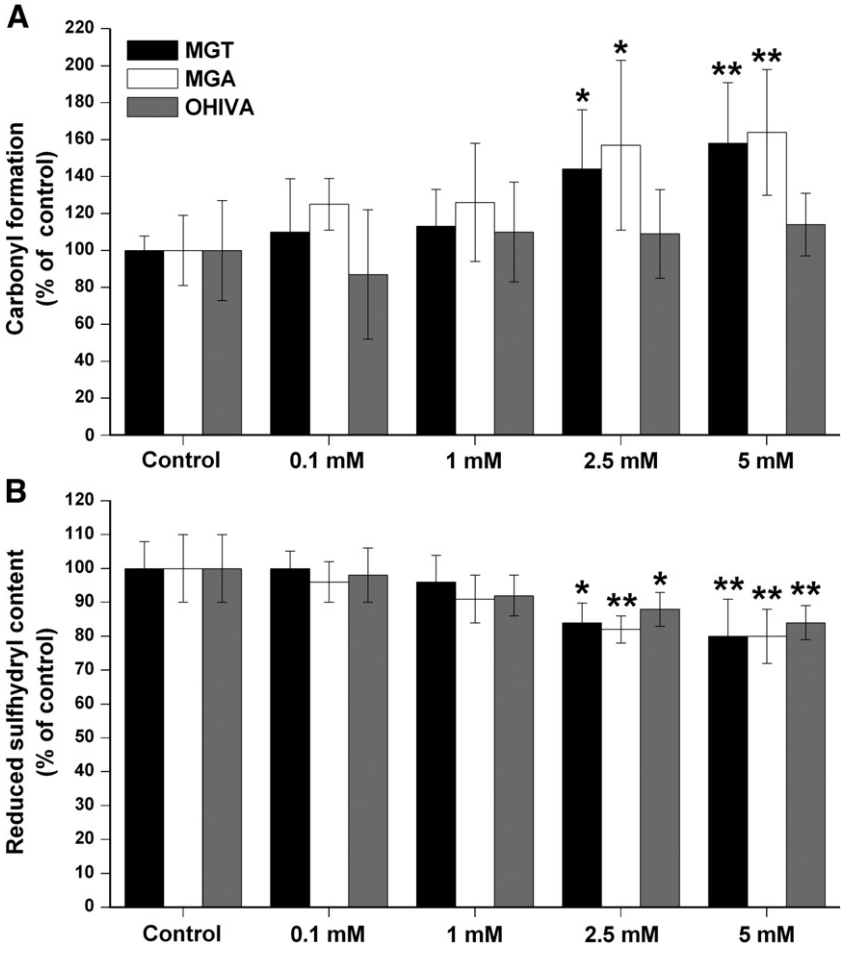


Fig. 5. In vitro effect of 3-methylglutaconate (MGT), 3-methylglutarate (MGA) and 3-hydroxyisovalerate (OHIVA) on carbonyl (A) and reduced sulfhydryl (B) content in rat cerebral cortex supernatants. Cortical supernatants were incubated during 60 min in the presence of these metabolites at concentrations ranging from 0.1 to 5.0 mM. Values are means±standard deviation for four to seven independent experiments performed in triplicate and are expressed as percentage of controls (Controls: Carbonyl formation (A): MGT: 0.7±0.027 nmol/mg of protein; MGA: 0.69±0.10 nmol/mg of protein; OHIVA: 0.85±0.23 nmol/mg of protein; Sulfhydryl content (B): MGT: 42.9±3.4 nmol/mg protein; MGA: 58.4±5.8 nmol/mg protein; OHIVA: 58.4±5.8 nmol/mg protein). *$P<0.05$; **$P<0.01$, compared to controls (Duncan multiple range test).

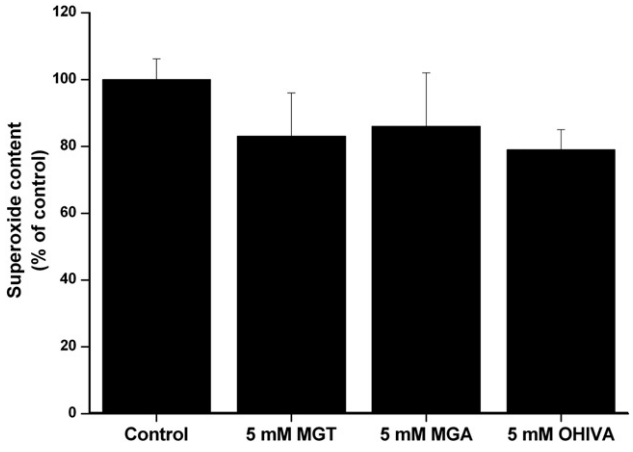


Fig. 6. In vitro effect of 3-methylglutaconate (MGT), 3-methylglutarate (MGA) and 3-hydroxyisovalerate (OHIVA) on superoxide generation in submitochondrial particles from rat cerebral cortex. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as percentage of controls (Control: 3.94 ± 0.24 nmol/min. mg of protein) (Duncan multiple range test).

Results

MGT, MGA and OHIVA induce lipid peroxidation in rat cortical supernatants

Fig. 2 shows that TBA-RS values were significantly increased in cortical supernatants exposed for 1 h to MGT [A: $F_{(4,20)}$= 24.05; $P<0.001$], MGA [B: $F_{(4,15)}=18.24$; $P<0.01$] and OHIVA [C: $F_{(4,20)}=7.71$; $P<0.01$] (up to 45% for MGT) in a dose-dependent manner [A: $\beta=0.898$; $P<0.001$; B: $\beta=0.899$; $P<0.001$; C: $\beta=0.765$; $P<0.001$]. Similarly, chemiluminescence was significantly enhanced in cortical supernatants by MGT [A: $F_{(4,30)}=3.338$; $P<0.05$], MGA [B: $F_{(4,15)}=11.64$; $P<0.001$] and OHIVA [C: $F_{(4,15)}=12.82$; $P<0.001$] (up to 34% for OHIVA) in a concentration-dependent fashion [A: $\beta=0.538$; $P<0.01$; B: $\beta=0.771$; $P<0.001$; C: $\beta=0.82$; $P<0.001$] (Fig. 2). These results indicate that these metabolites induced lipid oxidative damage.

Considering that MGT is the major metabolite accumulating in MGTA and that MGT provoked the highest increase in TBA-

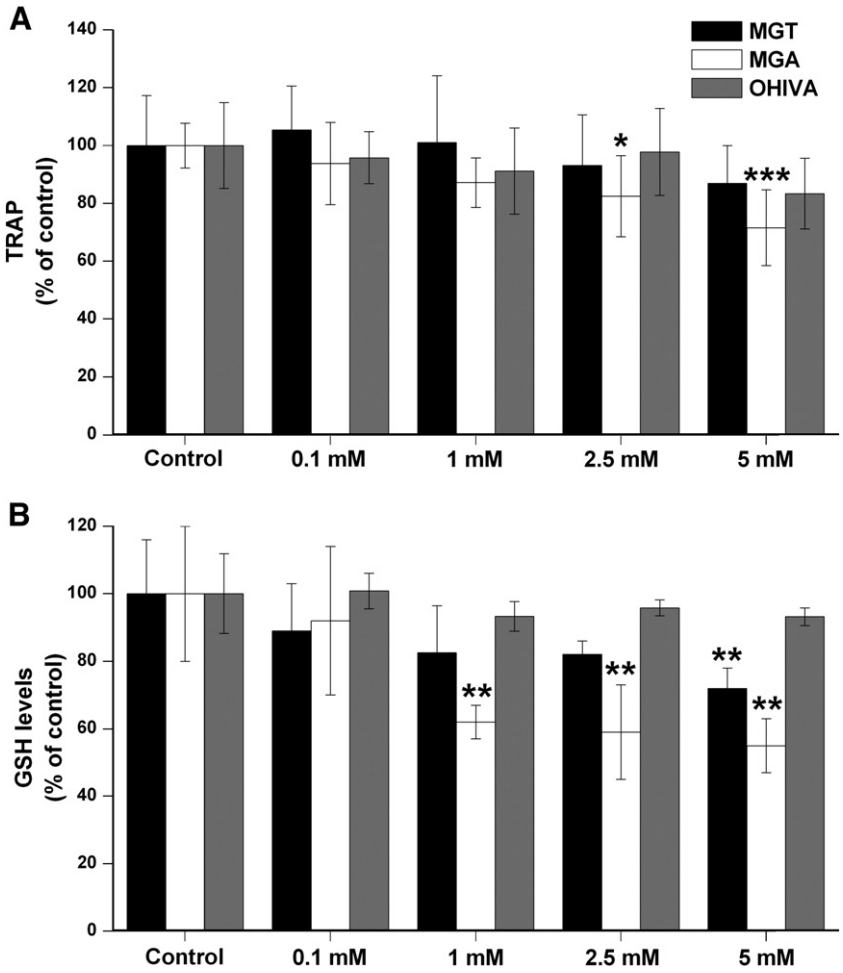


Fig. 7. In vitro effect of 3-methylglutaconate (MGT), 3-methylglutarate (MGA) and 3-hydroxyisovalerate (OHIVA) on the non-enzymatic antioxidant defenses total radical-trapping antioxidant potential (TRAP) (A) and glutathione (GSH) levels (B) in rat cerebral cortex supernatants. Values are means ± standard deviation for four to six independent experiments performed in triplicate and are expressed as percentage of controls (Controls: TRAP values (A): MGT: 22.1 ± 2.3 nmol/mg protein; MGA: 24.0 ± 1.9 nmol/mg of protein; OHIVA: 15.6 ± 1.8 nmol/mg protein; GSH levels (B): MGT: 7.64 ± 1.54 nmol/mg protein; MGA: 7.64 ± 1.54 nmol/mg protein; OHIVA: 12.4 ± 1.59 nmol/mg of protein). *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared to controls (Duncan multiple range test).

RS levels (45%), we evaluated the role of antioxidants on MGT-induced increase on TBA-RS levels. Cortical supernatants were pre-incubated with the antioxidants TRO (α-tocopherol; 1.5 μM), GSH (100 μM), L-NAME (500 μM), MEL (200 μM) or the combination of SOD plus CAT (2.5 mU/mL each) and 5.0 mM MGT. It can be observed that none antioxidant was able to prevent MGT-induced in vitro lipoperoxidation, except for melatonin that significantly attenuated the lipid oxidative damage induced by MGT [$F_{(3,16)}=48.23$; $P<0.001$] (Fig. 3).

Thereafter, we tested whether higher doses of TRO (5 μM), MEL (1000 μM) or the combination of SOD and CAT (10 mU/mL each) could prevent the augmented levels of TBA-RS elicited by MGT. Cortical supernatants were pre-incubated for 15 min with these antioxidants before the addition of 5.0 mM MGT. The results show that, at the higher doses, all tested antioxidants (TRO, MEL and SOD plus CAT) totally prevented the lipid peroxidation induced by MGT (TRO: [$F_{(2,9)}=18.21$; $P<0.001$]; MEL: [$F_{(2,9)}=21.03$; $P<0.001$]; SOD+CAT: [$F_{(2,15)}=19.35$; $P<0.001$]) (Fig. 4).

MGT and MGA induce protein oxidative damage

Next, we evaluated the in vitro effect of MGT, MGA and OHIVA on carbonyl formation and sulfhydryl oxidation in cerebral cortex from young rats. MGT [$F_{(4,24)}=5.444$; $P<0.01$] and MGA [$F_{(4,22)}=4.077$; $P<0.01$] provoked a significant increase in carbonyl formation (up to 58% and 64%, respectively) (Fig. 5A). Moreover, MGT augmented carbonyl formation in a dose-dependent fashion [$\beta=0.663$; $P<0.001$]. In contrast, OHIVA did not significantly alter carbonyl content (Fig. 5A).

Furthermore, we observed that MGT [$F_{(4,15)}=5.577$; $P<0.01$], MGA [$F_{(4,15)}=5.607$; $P<0.01$] and OHIVA [OHIVA: $F_{(4,15)}=3.7$; $P<0.01$] significantly induced sulfhydryl oxidation in a dose-dependent manner [MGT: $\beta=-0.738$; $P<0.001$] [MGA: $\beta=-0.712$; $P<0.001$] [OHIVA: $\beta=-0.669$; $P<0.01$] (Fig. 5B).

Superoxide content is not affected by MGT, MGA and OHIVA

We also verified that MGT, MGA and OHIVA did not alter superoxide content in submitochondrial particles when these preparations were exposed for 1h to these metabolites (Fig. 6).

MGA and MGT diminish non-enzymatic antioxidant defenses

The non-enzymatic antioxidant defenses were also investigated by assessing TRAP and GSH levels. Fig. 7 shows that MGA significantly diminished TRAP (up to 20%) [A: $F_{(4,20)}=7.38$; $P<0.001$] and GSH levels (up to 45%) [B: $F_{(4,15)}=7.16$; $P<0.01$] in a dose-dependent manner [TRAP: $\beta=-0.756$; $P<0.001$] [GSH: $\beta=0.65$; $P<0.01$] and MGT significantly reduced GSH levels (up to 38%) [B: $F_{(4,19)}=3.98$; $P<0.01$]. In contrast, OHIVA did not significantly alter these parameters.

We also tested whether TRO, MEL and SOD plus CAT could prevent MGA-decrease of GSH levels in cortical supernatants. We observed that 1.0 mM melatonin [$F_{(2,9)}=13.86$; $P<0.01$] and 10 μM α-tocopherol (TRO) [$F_{(2,9)}=12.22$; $P<0.01$], but not SOD plus CAT (even at a concentration as high as 15 mU/mL) were able to prevent the MGA-induced decrease of reduced glutathione (GSH) levels in cortical supernatants (Fig. 8).

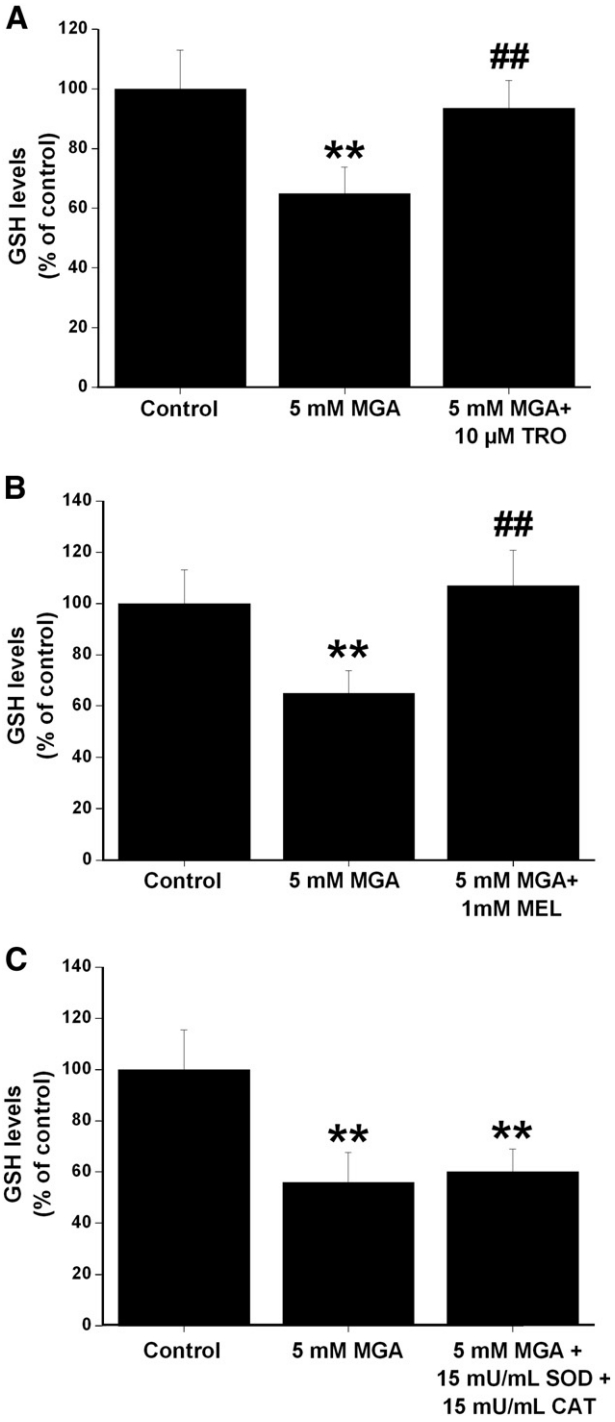


Fig. 8. Effect of increasing concentrations of antioxidants on 3-methylglutarate (MGA) reduction of GSH levels in rat cerebral cortex supernatants. Cortical supernatants were pre-incubated for 15 min with the antioxidants Trolox (TRO, 10 μM) (A), melatonin (MEL; 1000 μM) (B) or the combination of catalase (CAT) plus superoxide dismutase (SOD) (15 mU/mL of each enzyme) (C) before the addition of 5.0 mM MGA. Values are means±standard deviation of four to six independent experiments performed in triplicate and are expressed as percentage of controls (Controls [nmol/mg protein]: A: 5.83±0.76; B: 5.83±0.76; C: 5.35±0.82). $^{**}P<0.01$, compared to controls; $^{\#\#}P<0.01$, compared to 5.0 mM MGA (Duncan multiple range test).

Table 1
Effect of 3-methylglutaconate (MGT), 3-methylglutarate (MGA) and 3-hydroxyisovalerate (OHIVA) on commercial reduced glutathione (GSH) and on purified mitochondrial membrane protein-bound sulfhydryl groups (PBSG) from rat cortical supernatants

GSH oxidation	
Control	100±1
5.0 mM MGT	106±1
5.0 mM MGA	107±5
5.0 mM OHIVA	99±13
250 μM NEM	71.5±31***
Mitochondrial PBSG oxidation	
Control	100±21.6
5.0 mM MGT	95.2±7.4
5.0 mM MGA	87.7±4.3
5.0 mM OHIVA	99.2±6
250 μM PHMB	62±29***

PHMB = *p*-hydroxymercuribenzoic acid; NEM = *N*-ethylmaleimide (positive controls). Values are means±standard deviation for three to five independent experiments performed in triplicate and are expressed as percentage of controls. GSH (200 μM) oxidation was measured in the absence of brain tissue. Controls: *GSH oxidation:* 1052±42 fluorescence units; *Mitochondrial PBSG oxidation*: 74.0±16 nmol/mg of protein. ***$P<0.001$ compared to controls (Duncan multiple range test).

MGT, MGA and OHIVA per se do not behave as direct oxidants

Finally, we investigated whether MGT, MGA and OHIVA could by itself act as oxidant agents. We therefore exposed brain purified mitochondrial membrane protein-bound sulfhydryl groups (PBSG) and a commercial glutathione (GSH) solution to 5.0 mM MGT, MGA or OHIVA in the absence of tissue supernatants. Table 1 shows that none of these metabolites significantly modified GSH or PBSG, whereas *N*-ethylmaleimide (NEM, 250 μM) and *p*-hydroxymercuribenzoic acid (PHMB, 250 μM) exposition (positive control) markedly oxidized GSH and PBSG, respectively. The data indicate that MGT, MGA and OHIVA do not behave as direct oxidant agents.

Discussion

Individuals affected by 3-methylglutaconic aciduria (MGTA) predominantly accumulate 3-methylglutaconic acid (MGT) and 3-methylglutaric acid (MGA) in their tissues and body fluids, whereas 3-hydroxyisovaleric acid (OHIVA) is also accumulated when this disorder is caused by 3-methylglutaconyl-CoA hydratase deficiency (MGTA type I). Although the patients commonly present neurologic manifestations, the pathophysiology of their symptoms is poorly established. However, it could be presumed that the increased tissue concentrations of MGT, MGA and OHIVA are associated with their neurologic symptoms, since they become worse during episodes of metabolic decompensation, in which there is a dramatic increase of the concentrations of these organic acids.

In this context, considering that oxidative stress has been shown to be involved in the pathophysiology of common neurodegenerative disorders (Behl and Moosmann, 2002; Bogdanov et al., 2001; Stoy et al., 2005; Berg and Youdim, 2006; Mancuso et al., 2006), including some inborn errors of metabolism (Latini et al., 2003a,b, 2005; de Oliveira Marques et al., 2003; Barschak et al., 2006; Sgaravatti et al., 2007), in the present investigation we evaluated the in vitro effects of MGT, MGA and OHIVA on important parameters of oxidative stress in cerebral cortex of young rats. Initially, we demonstrated that these metabolites significantly increased chemiluminescence and TBA-RS levels. Light in the chemiluminescence assay can arise mainly from excited carbonyls, $O_2^{\cdot-}$, $ONOO^-$ and from peroxidizing lipids (Halliwell and Gutteridge, 2007a) as a result of increased oxygen and nitrogen free radical production. On the other hand, TBA-RS measurement reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge, 2007a). Therefore, the augmented values of these parameters provoked by MGT, MGA and OHIVA indicate an induction of lipid oxidative damage by these metabolites. We also observed that the in vitro lipid peroxidation provoked by MGT was totally prevented by high doses of the free radical scavengers TRO (α-tocopherol), MEL and the combination of SOD plus CAT, although smaller doses of TRO, SOD plus CAT, as well as GSH and L-NAME were not able to change the MGT-induced increase on TBA-RS values. Furthermore, relatively low concentrations of MEL significantly attenuated the increase of TBA-RS measurement provoked by MGT. We also verified that these metabolites did not elicit superoxide generation in submitochondrial particle preparations. Taken together these observations, it may be presumed that peroxyl and hydroxyl radicals, as well as hydrogen peroxide, which are scavenged by these antioxidants (Reiter et al., 1997a,b, 2001; Anisimov et al., 2006), are involved in the pro-oxidant effects of MGT.

We also observed that MGT and MGA, but not OHIVA, increased protein carbonyl formation, whereas MGT, MGA and OHIVA significantly elicited sulfhydryl oxidation. On the other hand, since these metabolites were not able to directly oxidize thiol groups from commercial (GSH) and brain purified mitochondrial preparations, it is presumed that MGT, MGA and OHIVA induced protein oxidative damage via reactive species generation (Reznick and Packer, 1993; Levine, 2002).

With regard to the antioxidant defense system, MGA markedly decreased the overall content of non-enzymatic antioxidants (TRAP values) and GSH values, the main naturally-occurring antioxidant. Furthermore, MGT also reduced GSH levels, but this effect was of lesser magnitude than that provoked by MGA. Conversely, OHIVA did not alter these non-enzymatic antioxidant defenses. Since these parameters are used to evaluate the non-enzymatic antioxidant capacity of a tissue to prevent the damage associated to free radical processes, it may be presumed that the rat cortical non-enzymatic antioxidant defenses were compromised by these metabolites, probably secondarily to reactive species generation (Lissi et al., 1995; Evelson et al., 2001; Halliwell and Gutteridge, 2007b). We also verified that TRO and MEL, but not the combination of SOD plus CAT, were able to prevent the MGA-decrease of GSH levels in cortical supernatants, indicating that peroxyl and hydroxyl radicals are the main species involved in this effect.

At this point it should be emphasized that the central nervous system is highly sensitive to oxidative stress due to its high oxygen consumption, its high iron and lipid contents, especially polyunsaturated fatty acids, and the low activity of antioxidant defenses (Halliwell and Gutteridge, 2007b). Considering that oxidative stress can be elicited by the imbalance between free radical production and antioxidant defenses and since the metabolites accumulating in MGTA provoked lipid and protein damage, as well as a reduction of the antioxidant defenses, it might be postulated that oxidative stress is induced by the major metabolites accumulating in this group of disorders.

We cannot at present ascertain the pathophysiological relevance of our data since to our knowledge brain concentrations of the metabolites that accumulate in MGTA affected patients are not yet established. However, the concentrations of these substances resulting in significant alterations of the parameters investigated were relatively high, possibly suprapathological. On the other hand, it has been proposed that the concentrations of organic acids, particularly dicarboxylic acids (including MGT and MGA) accumulating in various organic acidemias are much higher in the brain as compared to the serum because these compounds are thought to be trapped in neural cells (Hoffmann et al., 1993, 1994; Sauer et al., 2006; Stellmer et al., 2007).

In conclusion, we report for the first time a possible deleterious influence of the metabolites accumulating in MGTA in the brain of young rats causing oxidative damage. Further in vitro and mainly in vivo studies are required to clarify the role of oxidative stress in the pathophysiology of MGTA. In case the in vitro effects detected in the present study also occur in vivo, it is tempted to speculate that they might be involved, at least in part, to the neurological dysfunction found in MGTA. In this scenario, it may be proposed that the administration of antioxidants could be considered as a potential adjuvant therapy for the affected patients.

Acknowledgements

This work was supported in part by grants from FAPERGS, PRONEX II and CNPq-Brazil.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lfs.2007.12.024.

References

Anikster, Y., Kleta, R., Shaag, A., Gahl, W.A., Elpeleg, O., 2001. Type III 3-methylglutaconic aciduria (optic atrophy plus syndrome, or Costeff optic atrophy syndrome): identification of the OPA3 gene and its founder mutation in Iraqi Jews. American Journal of Human Genetics 69 (6), 1218–1224.

Anisimov, V.N., Popovich, I.G., Zabezhinski, M.A., Anisimov, S.V., Vesnushkin, G.M., Vinogradova, I.A., 2006. Melatonin as antioxidant, geroprotector and anticarcinogen. Biochimica et Biophysica Acta 1757 (5-6), 573–589.

Arn, P., Funanage, V.L., 2006. 3-Methylglutaconic aciduria disorders: the clinical spectrum increases. Journal of Pediatric Hematology/Oncology 28 (2), 62–63.

Barschak, A.G., Sitta, A., Deon, M., Oliveira, M.H., Haeser, A., Dutra-Filho, C.S., Wajner, M., Vargas, C.R., 2006. Evidence that oxidative stress is increased in plasma from patients with maple syrup urine disease. Metabolic Brain Disease 21 (4), 279–286.

Barth, P.G., Valianpour, F., Bowen, V.M., Jam, J., Duran, M., Vaz, F.M., Wanders, R.J.A., 2004. X-linked cardioskeletal myopathy and neutropenia (Barth syndrome): an update. American Journal of Medical Genetics 126 (4), 39–354.

Behl, C., Moosmann, B., 2002. Oxidative nerve cell death in Alzheimer's disease and stroke: antioxidants as neuroprotective compounds. Biological Chemistry 383 (3-4), 521–536.

Berg, D., Youdim, M.B., 2006. Role of iron in neurodegenerative disorders. Topics in Magnetic Resonance Imaging 17 (1), 5–17.

Besley, G.T., Lendon, M., Broadhead, D.M., Till, J., Heptinstall, L.E., Phillips, B., 1995. Mitochondrial complex deficiencies in a male with cardiomyopathy and 3-methylglutaconic aciduria. Journal of Inherited Metabolic Disease 18 (2), 221–223.

Bogdanov, M.B., Andreassen, O.A., Dedeoglu, A., Ferrante, R.J., Beal, M.F., 2001. Increased oxidative damage to DNA in a transgenic mouse of Huntington's disease. Journal of Neurochemistry 79 (6), 1246–1249.

Bione, S., D, 'Adamo, P., Maestrini, E., Gedeon, A.K., Bolhuis, P.A., Toniolo, D., 1996. A novel x-linked gene, G4.5. is responsible for Barth syndrome. Nature Genetics 12 (4), 385–389.

Browne, R.W., Armstrong, D., 1998. Reduced glutathione and glutathione disulfide. Methods in Molecular Biology 108, 347–352.

de Oliveira Marques, F., Hagen, E.K., Pederzolli, C.D., Sgaravatti, A.M., Durigon, K., Testa, C.G., Wannmacher, C.W., Wyse, A.S., Wajner, M., Dutra-Filho, C.S., 2003. Glutaric acid induces oxidative stress in brain of young rats. Brain Research 964 (1), 153–158.

Di Rosa, G., Deodato, F., Loupatty, F.J., Rizzo, C., Carrozzo, R., Santorelli, F.M., Boenzi, S., D, 'Amico, A., Tozzi, G., Bertini, E., Maiorana, A., Wanders, R.J.A., Dionisi-Vici, C., 2006. Hypertrophic cardiomyopathy, cataract, developmental delay, lactic acidosis: a novel subtype of 3-methylglutaconic aciduria. Journal of Inherited Metabolic Disease 29 (4), 546–550.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods in Enzymology 186, 407–421.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., Lissi, E., 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Archives of Biochemistry and Biophysics 388 (2), 261–266.

Gibson, K.M., Sherwood, W.G., Hoffmann, G.F., Stumpf, D.A., Dianzani, I., Schutgens, R.B., Barth, P.G., Weismann, U., Bachmann, C., Schrynemackers-Pitance, P., et al., 1991. Phenotypic heterogeneity in the syndromes of 3-methylglutaconic aciduria. The Journal of Pediatrics 118 (6), 885–890.

Gibson, K.M., Elpeleg, O.N., Jacobs, C., Costeff, H., Kelley, R.I., 1993. Multiple syndromes of 3-methylglutaconic aciduria. Pediatric Neurology 9 (2), 120–123.

Gonzalez-Flecha, B., Llesuy, S., Boveris, A., 1991. Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver, and muscle. Free Radical Biology & Medicine 10 (2), 93–100.

Gunay-Aygun, M., 2005. 3-Methylglutaconic aciduria: a common biochemical marker in various syndromes with diverse clinical features. Molecular and Genetics Metabolism 84, 1–3.

Halliwell, B., Gutteridge, J.M.C., 2007a. Measurement of reactive species. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. Oxford University Press, Oxford, pp. 268–340.

Halliwell, B., Gutteridge, J.M.C., 2007b. Cellular responses to oxidative stress: adaptation, damage, repair, senescence ad death. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. Oxford University Press, Oxford, pp. 187–267.

Hoffmann, G.F., Meier-Augenstein, W., Stockler, S., Surtees, R., Rating, D., Nyhan, W.L., 1993. Physiology and pathophysiology of organic acids in cerebrospinal fluid. Journal of Inherited Metabolic Disease 16 (4), 648–669.

Hoffmann, G.F., Gibson, K.M., Trefz, F.K., Nyhan, W.L., Bremer, H.J., Rating, D., 1994. Neurological manifestations of organic acid disorders. European Journal of Pediatrics 53, S94–S100.

Ibel, H., Endres, W., Hadorn, H.B., Deufel, T., Paetske, I., Duran, M., Kennaway, N.G., Gibson, K.M., 1993. Multiple respiratory chain abnormalities associated with hypertrophic cardiomyopathy and 3-methylglutaconic aciduria. European Journal of Pediatrics 152 (8), 665–670.

Ijlst, L., Loupaty, F.J., Ruiter, J.P.N., Duran, M., Lehnert, W., Wanders, R.J.A., 2002. 3-Methylglutaconic aciduria type I is caused by mutations in AUH. American Journal of Human Genetics 71 (6), 1463–1466.

Kowaltowski, A.J., Vercesi, A.E., Castilho, R.F., 1997. Mitochondrial membrane protein thiol reactivity with *N*-ethylmaleimide or mersalyl is modified by Ca2+: correlation with mitochondrial permeability transition. Biochimica et Biophysica Acta 1318 (3), 395–402.

Latini, A., Scussiato, K., Rosa, R.B., Leipnitz, G., Llesuy, S., Klein, A.B., Dutra-Filho, C.S., Wajner, M., 2003a. Induction of oxidative stress by L-2-hydroxyglutaric acid in rat brain. Journal of Neuroscience Research 74 (1), 103–110.

Latini, A., Scussiato, K., Rosa, R.B., Llesuy, S., Bello-Klein, A., Dutra-Filho, C.S., Wajner, M., 2003b. D-2-hydroxyglutaric acid induces oxidative stress in cerebral cortex of young rats. The European Journal of Neuroscience 17 (10), 2017–2022.

Latini, A., Scussiato, K., Leipnitz, G., Dutra-Filho, C.S., Wajner, M., 2005. Promotion of oxidative stress by 3-hydroxyglutaric acid in rat stritum. Journal of Inherited Metabolic Disease 28 (1), 57–67.

Levine, R.L., 2002. Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radical Biology & Medicine 32 (9), 790–796.

Lissi, E., Pascual, C., Del Castillo, M.D., 1992. Luminol luminescence induced by 2,2-azo-bis-(2-amidinopropane) thermolysis. Free Radical Research Communications 17 (5), 299–311.

Lissi, E., Salim-Hanna, M., Pascual, C., del Castillo, M.D., 1995. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. Free Radical Biology & Medicine 18 (2), 153–158.

Lowry, O.H., Rosebrough, N.J., Lewis-Farr, A., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193 (1), 265–275.

Mancuso, M., Coppede, F., Migliore, L., Siciliano, G., Murri, L., 2006. Mitochondrial dysfunction, oxidative stress and neurodegeneration. Journal of Alzheimer's Disease 10 (1), 59–73.

Poderoso, J.J., Carreras, M.C., Lisdero, C., Riobó, N., Schöpfer, F., Boveris, A., 1996. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. Archives of Biochemistry and Biophysics 328 (1), 85–92.

Reiter, R.J., Carneiro, R.C., Oh, C.S., 1997a. Melatonin in relation to cellular antioxidative defense mechanisms. Hormone and Metabolic Research 29 (8), 363–372.

Reiter, R., Tang, L., Garcia, J.J., Munoz-Hoyos, A., 1997b. Pharmacological actions of melatonin in oxygen radical pathophysiology. Life Sciences 60 (25), 2255–2271.

Reiter, R.J., Tan, D.X., Manchester, L.C., Qi, W., 2001. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. Cell Biochemistry and Biophysics 34 (2), 237–256.

Reznick, A.Z., Packer, L., 1993. Free radicals and antioxidants in muscular neurological diseases and disorders. In: Poli, G., Albano, E., Dianzani, U.M. (Eds.), Free Radicals: from Basic Science to Medicine. Birkhauser Verlag, Basel, pp. 425–437.

Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods in Enzymology 233, 357–363.

Ribeiro, C.A.J., Balestro, F., Grando, V., Wajner, M., 2007. Isovaleric acid reduces Na+,K+-ATPase activity in synaptic membranes from cerebral cortex of young rats. Cellular and Molecular Neurobiology 27 (4), 529–540.

Ruesch, S., Krühenbühl, S., Kleinle, S., Liechti-Gallati, S., Schaffner, T., Wermuth, B., Weber, J., Wiesmann, U.N., 1996. Combined 3-methylglutaconic and 3-hydroxy-3-methylglutaric aciduria with endocardial fibroelastosis and dilatative cardiomyopathy in male and female siblings with partial deficiency of complex II/III in fibroblasts. Enzyme Protein 49 (5-6), 321–329.

Sauer, S.W., Okun, J.G., Fricker, G., Mahringer, A., Müller, I., Crnic, L.S., Mühlhausen, C., Hoffmann, G.F., Hörster, F., Goodman, S.I., Harding, C.O., Koeller, D.M., Kölker, S., 2006. Intracerebral accumulation of glutaric and 3-hydroxyglutaric acids secondary to limited flux across the blood-brain barrier constitute a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. Journal of Neurochemistry 97 (3), 899–910.

Sgaravatti, A.M., Sgarbi, M.B., Testa, C.G., Durigon, K., Pederzolli, C.D., Prestes, C.C., Wyse, A.T., Wannmacher, C.M., Wajner, M., Dutra-Filho, C.S., 2007. γ-Hydroxybutyric acid induces oxidative stress in cerebral cortex of young rats. Neurochemistry International 50 (3), 564–570.

Shoji, Y., Takahashi, T., Sawaishi, Y., Ishida, A., Matsumori, M., Shoji, Y.A., Enoki, M., Watanabe, H., Takada, G., 1999. 3-Methylglutaconic aciduria type I: clinical heterogeneity as a neuroetabolic disease. Journal of Inherited Metabolic Disease 22 (1), 1–8.

Stellmer, F., Keyser, B., Burckhardt, B.C., Koepsell, H., Streichert, T., Glatzel, M., Jabs, S., Thiem, J., Herdering, W., Koeller, D.M., Goodman, S.I., Lukacs, Z., Ullrich, K., Burckhardt, G., Braulke, T., Muhlhausen, C., 2007. 3-Hydroxyglutaric acid is transported via the sodium-dependent dicarboxylate transporter NaDC3. Journal of Molecular Medicine 85 (7), 763–770.

Stoy, N., Mackay, G.M., Forrest, C.M., Christofides, J., Egerton, M., Stone, T.W., Darlington, L.G., 2005. Tryptophan metabolism and oxidative stress in patients with Huntington's disease. Journal of Neurochemistry 93 (3), 611–623.

Sweetman, L., Williams, 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.

Wortmann, S., Rodenburg, R.J.T., Huizing, M., Loupatty, F.J., de Koning, T., Kluijtmans, L.A.J., Engelke, U., Wevers, R., Smeitink, J.A.M., Morava, E., 2006. Association of 3-methylglutaconic aciduria with sensori-neural deafness, encephalopathy, and Leigh-like syndrome (MEGDEL association) in four patients with a disorder of the oxidative phosphorylation. Molecular Genetics and Metabolism 88 (1), 47–52.

Capítulo III

Striatum is more vulnerable to oxidative damage induced by the metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency as compared to liver

Guilhian Leipnitz, Bianca Seminotti, Carolina G. Fernandes, Alexandre U. Amaral, Ana Paula Beskow, Lucila de B. da Silva, Ângela Zanatta, César A. J. Ribeiro, Carmen R. Vargas, Moacir Wajner.

Artigo científico publicado no periódico

International Journal of Developmental Neuroscience

Int. J. Devl Neuroscience 27 (2009) 351–356

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

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

Striatum is more vulnerable to oxidative damage induced by the metabolites accumulating in 3-hydroxy-3-methylglutaryl-CoA lyase deficiency as compared to liver

Guilhian Leipnitz [a], Bianca Seminotti [a], Carolina G. Fernandes [a], Alexandre U. Amaral [a], Ana Paula Beskow [a], Lucila de B. da Silva [a], Ângela Zanatta [a], César A.J. Ribeiro [a], Carmen R. Vargas [a], Moacir Wajner [a,b,c,*]

[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, RS, Brazil
[c] Universidade Luterana do Brasil, Canoas, RS, Brazil

ARTICLE INFO

Article history:
Received 5 January 2009
Received in revised form 11 February 2009
Accepted 3 March 2009

Keywords:
3-Hydroxy-3-methylglutaric aciduria
Oxidative stress
Striatum
Liver

ABSTRACT

The present work investigated the *in vitro* effects of 3-hydroxy-3-methylglutarate, 3-methylglutarate, 3-methylglutaconate and 3-hydroxyisovalerate, which accumulate in 3-hydroxy-3-methylglutaric aciduria, on important parameters of oxidative stress in striatum and liver of young rats, tissues that are injured in this disorder. Our results show that all metabolites induced lipid peroxidation (thiobarbituric acid-reactive substances increase) and decreased glutathione levels in striatum, whereas 3-hydroxy-3-methylglutarate, besides inducing the strongest effect, also altered thiobarbituric acid-reactive substances and glutathione levels in the liver. Furthermore, 3-hydroxy-3-methylglutarate, 3-methylglutarate and 3-methylglutaconate oxidized sulfhydryl groups in the striatum, but not in the liver. Our data indicate that 3-hydroxy-3-methylglutarate behaves as a stronger pro-oxidant agent compared to the other metabolites accumulating in 3-hydroxy-3-methylglutaric aciduria and that the striatum present higher vulnerability to oxidative damage relatively to the liver.

3-Hydroxy-3-methylglutaric aciduria (HMGA; McKusick 246450) is an autosomal recessive branched chain organic aciduria caused by the deficiency of the enzyme 3-hydroxy-3-methylglutaryl-CoA lyase (HL; EC 4.1.3.4). This mitochondrial enzyme catalyses both leucine degradation and ketogenesis. Clinical presentation occurs within the first year of life. Acute episodes are common and characterized by vomiting, hypotonia, tachypnea, seizures, lethargy sometimes progressing to coma, metabolic acidosis with hypoketotic hypoglycemia, variable hyperammonaemia and elevation of plasma transaminases with hepatomegaly (Funghini et al., 2001; Sweetman and Williams, 2001; Vargas et al., 2008; Zafeiriou et al., 2007). Chronic symptomatology includes macrocephalia and variable developmental delay (Wysocki and Hahnel, 1986; Gibson et al., 1988, 1994; Lee et al., 1999; Funghini et al., 2001; Pospisilova et al., 2003; Zafeiriou et al., 2007).

The disorder is biochemically characterized by predominant tissue accumulation and high urinary excretion of large quantities of 3-hydroxy-3-methylglutarate (HMG), as well as 3-methylglutaconate (MGT), 3-methylglutarate (MGA), 3-hydroxyisovalerate (OHIVA) and 3-methylcrotonylglycine (Bonafe et al., 2000; Sweetman and Williams, 2001). The average concentration of urinary HMG, the major accumulating compound in HMGA patients, is about 1300 mmol/mol of creatinine, but it can rise to 11,000 mmol/mol of creatinine during metabolic crises (Sweetman and Williams, 2001). Smaller, but appreciable levels of glutarate, lactate, adipate and other dicarboxylic acids may also be excreted in the urine, especially during metabolic decompensation (Gibson et al., 1988). Recurrent crises occur, especially following prolonged fasting and infections characterized by increased catabolism.

The most common abnormalities found in the magnetic resonance neuroimaging involve multiple and marked coalescent lesions in periventricular subcortical white matter and arcuate fibers, most prominently in frontal or periatrial regions, which can be sometimes reversible after a leucine-free diet (Zafeiriou et al., 2007). Involvement of the caudate nucleus and the dentate nucleus

* Corresponding author at: Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel.: +55 51 33085571; fax: +55 51 21018010.
E-mail address: mwajner@ufrgs.br (M. Wajner).

0736-5748/$36.00 © 2009 ISDN. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijdevneu.2009.03.001

is also commonly observed in HMGA patients (van der Knaap et al., 1998; Yalcinkaya et al., 1999; Yylmaz et al., 2006).

Although neurological abnormalities are relatively pronounced in HMGA and hepatic dysfunction is found during metabolic crises, the mechanisms of tissue damage in this disorder are poorly known. However, it was recently described that HMG, MGT, MGA and OHIVA induce lipid and protein oxidative damage and reduce the non-enzymatic antioxidant defenses in cerebral cortex (Leipnitz et al., 2008a,b). Furthermore, the lipid peroxidation and glutathione levels decrease provoked by these metabolites were prevented by free radical scavengers, indicating the involvement of reactive species. The present study compares the *in vitro* influence of HMG, MGT, MGA and OHIVA on important parameters of oxidative stress, such as thiobarbituric acid-reactive substances (TBA-RS, lipid peroxidation), glutathione (GSH) levels (antioxidant defenses) and sulfhydryl group oxidation (protein oxidative damage) between striatum and liver from young rats. The main objective was therefore to evaluate the vulnerability of a central (striatum) and a peripheral (liver) tissue to the oxidative damage caused by the metabolites accumulating in HMGA in order to clarify the pathogenesis of this disorder.

1. Experimental procedures

1.1. Animals and reagents

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 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.

All chemicals were purchased from Sigma (St. Louis, MO, USA), except for MGT and OHIVA which were prepared by Dr. Ernesto Brunet with 99% purity (Universitat Autonoma de Madrid, Spain). The compounds were prepared on the day of the experiments in the incubation medium used for each technique and the pH was adjusted at 7.4. The final concentrations of the acids in the medium were 0.01–5.0 mM (striatum) or 1.0–5.0 mM (liver). Controls did not contain any of these metabolites in the incubation medium. TBA-RS and sulfhydryl group oxidation were measured with a double-beam Hitachi U-2001 spectrophotometer, whereas GSH levels were measured in a Hitachi F-2000 fluorescence spectrophotometer.

1.2. Sample preparation

On the day of the experiments the rats were sacrificed by decapitation without anaesthesia. The brain was rapidly excised on a Petri dish placed on ice, the olfactory bulbs, pons, medulla, hippocampus, cerebellum and cerebral cortex were discarded and the striatum was dissected. The liver was perfused with a cold solution of 0.9% sodium chloride. The striatum and the liver were weighed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 × *g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated at 37 °C for 1 h with HMG, MGT, MGA or OHIVA, or without any of these metabolites (controls). Immediately after incubation, aliquots were taken to measure TBA-RS, GSH and sulfhydryl oxidation.

We should emphasize that a total of 25 rats were used for the striatum experiments. Furthermore, different set of animals was used to search for the effect of each organic acid in the striatum because only about 30–40 mg of this tissue can be obtained from each animal. In contrast, we used liver (2000 mg can be obtained from each rat) from a total of 4–7 animals to run the experiments designed to test the effects of all acids in the liver.

1.3. Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL pre-treated supernatants and centrifuged at 3000 × *g* for 10 min. 300 μL of the supernatants were transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulphate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol/mg protein and represented as percentage of control.

1.4. Reduced glutathione (GSH) content

GSH concentrations were measured according to Browne and Armstrong (1998). Pre-treated supernatants were diluted in 20 volumes (1:20, v/v) of 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. 100 μL of this preparation were incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature during 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.001–1 mM) and the concentrations were calculated as nmol/mg protein and represented as percentage of control.

1.5. Sulfhydryl content

This assay is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). 30 μL of 10 mM DTNB prepared in 0.2 M potassium phosphate solution, pH 8.0, were added to 160 μL of the pre-treated to the supernatants. This was followed by a 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol/mg protein and represented as percentage of control.

1.6. Protein determination

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

1.7. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. Only significant F values are shown in the text. 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.

2. Results

First, we investigated the *in vitro* effect of HMG, MGT, MGA and OHIVA on TBA-RS levels in striatum and liver of 30-day-old rats. Fig. 1 shows that, among the various acids, HMG induced the highest degree of lipid peroxidation (up to 156%) *in vitro* at concentrations as low as 1 mM in the striatum [$F_{(5,18)} = 138.035$, $P < 0.001$], and also caused increased TBA-RS in the liver but to a lesser extent and only at 5 mM concentration [$F_{(2,12)} = 88.469$, $P < 0.001$]. MGT [$F_{(5,18)} = 18.858$, $P < 0.001$], MGA [$F_{(5,19)} = 3.588$, $P < 0.05$] and OHIVA [$F_{(5,18)} = 16.677$, $P < 0.001$] also provoked a lower but significant increase of TBA-RS values in the striatum, but not in the liver. The differences of control mean values found in the striatum were probably due to the normal individual variability since various sets of animals were used to test the effect of each organic acid in these experiments.

Next, we evaluated the effects of HMG, MGT, MGA and OHIVA on the concentrations of GSH. Again, HMG, at 1 and 5 mM concentrations, induced the strongest effect (up to 47%), compared to the other organic acids, reducing GSH levels in the striatum [$F_{(5,18)} = 11.203$, $P < 0.001$] and in the liver at 5 mM dose [$F_{(2,18)} = 6.436$, $P < 0.01$]. MGA also reduced the major non-enzymatic antioxidant defense in the striatum at 1 and 5 mM concentrations [$F_{(5,12)} = 10.856$, $P < 0.001$], whereas MGT [$F_{(5,18)} = 11.071$, $P < 0.001$] and OHIVA decreased this parameter only at 5 mM [$F_{(5,18)} = 5.293$, $P < 0.01$] with no effect on the liver (Fig. 2).

Finally, protein oxidative damage was assessed by measuring the effect of HMGA accumulating metabolites on sulfhydryl (thiol) oxidation in striatum and in liver. It can be seen in Fig. 3 that the organic acids utilized in the assays did not modify the sulfhydryl content in the liver, whereas HMG increased sulfhydryl oxidation

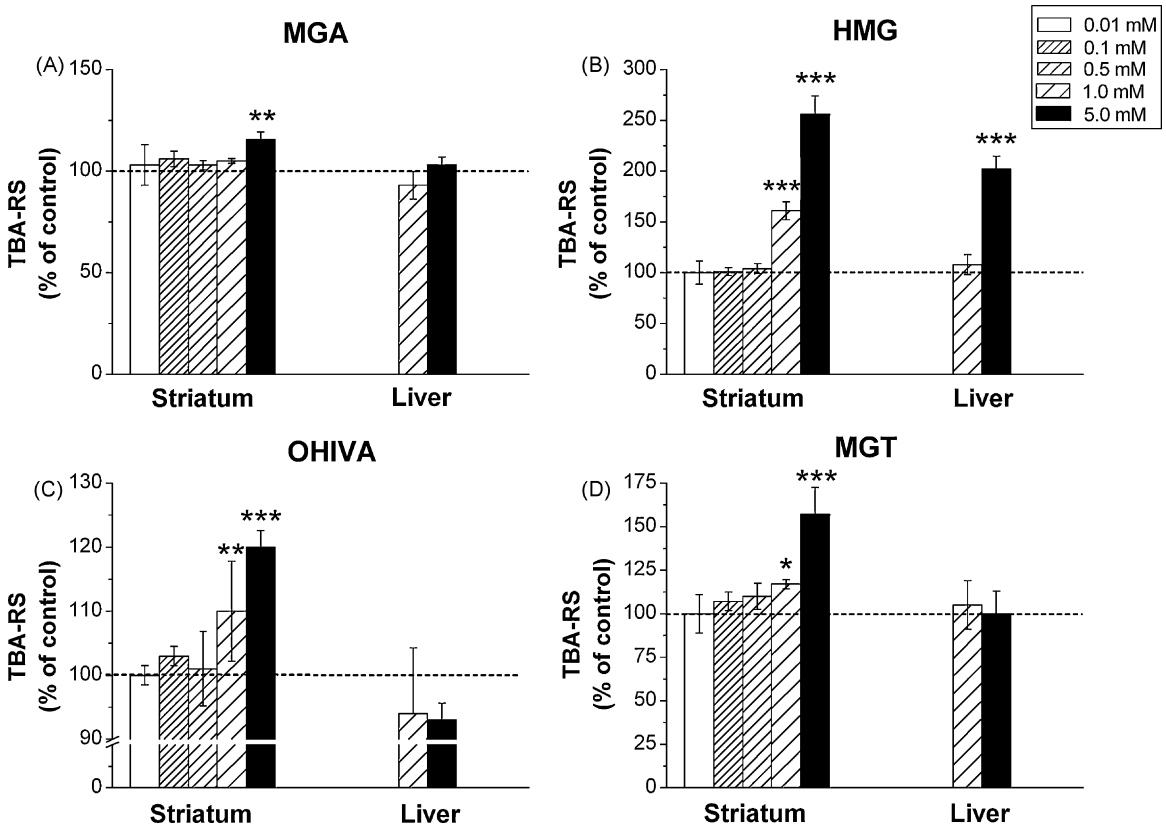


Fig. 1. Comparison between the *in vitro* effects of 3-methylglutaric acid (MGA; A), 3-hydroxy-3-methylglutaric acid (HMG; B), 3-hydroxyisovaleric acid (OHIVA; C) and 3-methylglutaconic acid (MGT; D) on thiobarbituric acid-reactive substances (TBA-RS) in striatum and liver of young rats. Data are represented as mean ± standard deviation of 3–7 animals for the striatum and 4–5 for the liver, performed in triplicate and expressed as percentage of controls (100%) (controls [nmol/mg of protein] – (A) striatum: 5.66 ± 0.39, liver: 0.81 ± 0.16; (B) striatum: 3.52 ± 0.42, liver: 0.8 ± 0.14; (C) striatum: 4.58 ± 1.58, liver: 0.85 ± 0.09; (D) striatum: 5.25 ± 0.68, liver: 0.9 ± 0.27). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test).

at 1 and 5 mM concentrations in the striatum [$F_{(5,18)} = 4.220$, $P < 0.05$]. Furthermore, MGT [$F_{(5,18)} = 6.577$, $P < 0.01$] and MGA [$F_{(5,18)} = 3.50$, $P < 0.01$] significantly oxidized thiol groups only in the striatum with a 5 mM dose, whereas OHIVA did not provoke any significant protein oxidative damage.

3. Discussion

Although neurological symptoms associated with brain abnormalities including macrocephaly and variable psychomotor delay are common features in HMGA and some patients present hepatic dysfunction, especially during metabolic decompensation (Ferris and Tien, 1993; Gordon et al., 1994; van der Knaap et al., 1998; Yalcinkaya et al., 1999; Yylmaz et al., 2006), the mechanisms underlying the brain and liver damage in HMGA are poorly known and the apparently higher vulnerability of the brain in this disorder is not established. In this context, oxidative stress has been shown to be involved in the pathophysiology of common neurological diseases, such as Parkinson's disease, Alzheimer's disease and Huntington's disease, as well as in various inborn errors of metabolism (Bogdanov et al., 2001; Behl and Moosmann, 2002; Stoy et al., 2005; Berg and Youdim, 2006; Mancuso et al., 2006; Latini et al., 2003a,b; Latini et al., 2005; de Oliveira Marques et al., 2003; Barschak et al., 2006; Sgaravatti et al., 2007). Furthermore, it has been recently demonstrated that the major metabolites accumulating in HMGA and 3-methylglutaconic aciduria induce lipid and protein oxidative damage and reduce the non-enzymatic antioxidant defenses in cerebral cortex of young rats (Leipnitz et al., 2008a,b).

Considering that significant abnormalities in the basal ganglia and liver alterations, but to a lesser extent, are found in patients affected by HMGA, in the present investigation we evaluated the *in vitro* effects of HMG, MGT, MGA and OHIVA on important parameters of oxidative stress in striatum and liver from young rats. We found that all tested metabolites significantly increased the levels of TBA-RS, which reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge, 2007a) in striatum from young rats, whereas only HMG induced lipid peroxidation in the liver. Furthermore, the oxidative effects elicited by HMG in the striatum were more severe than those caused by the other organic acids.

We also found that HMG, MGT, MGA and OHIVA markedly decreased GSH concentrations in the striatum, whereas only HMG decreased these levels in the liver. Again, HMG effects on the GSH concentrations were greater than those provoked by the other organic acids evaluated. GSH represents an important cytosolic antioxidant, helping to scavenge reactive oxygen species (ROS), protecting crucial cellular sulfhydryl groups and playing a key role in maintaining the intracellular redox state (Sarafian et al., 1996). 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 (Castoldi et al., 2001), it is possible that the significant reduction of GSH induced by the metabolites accumulating in HMGA may be related at least in part to the increased *in vitro* lipid peroxidation caused by these compounds. This is in line with the observations that GSH is considered an important defense against lipid oxidative damage eliminating hydrogen peroxide and peroxyl radicals formed during this process (Leipnitz et al., 2008a,b) and that supplementation of NAC, a GSH precursor, to cortical supernatants prevented *in vitro* lipid oxidative

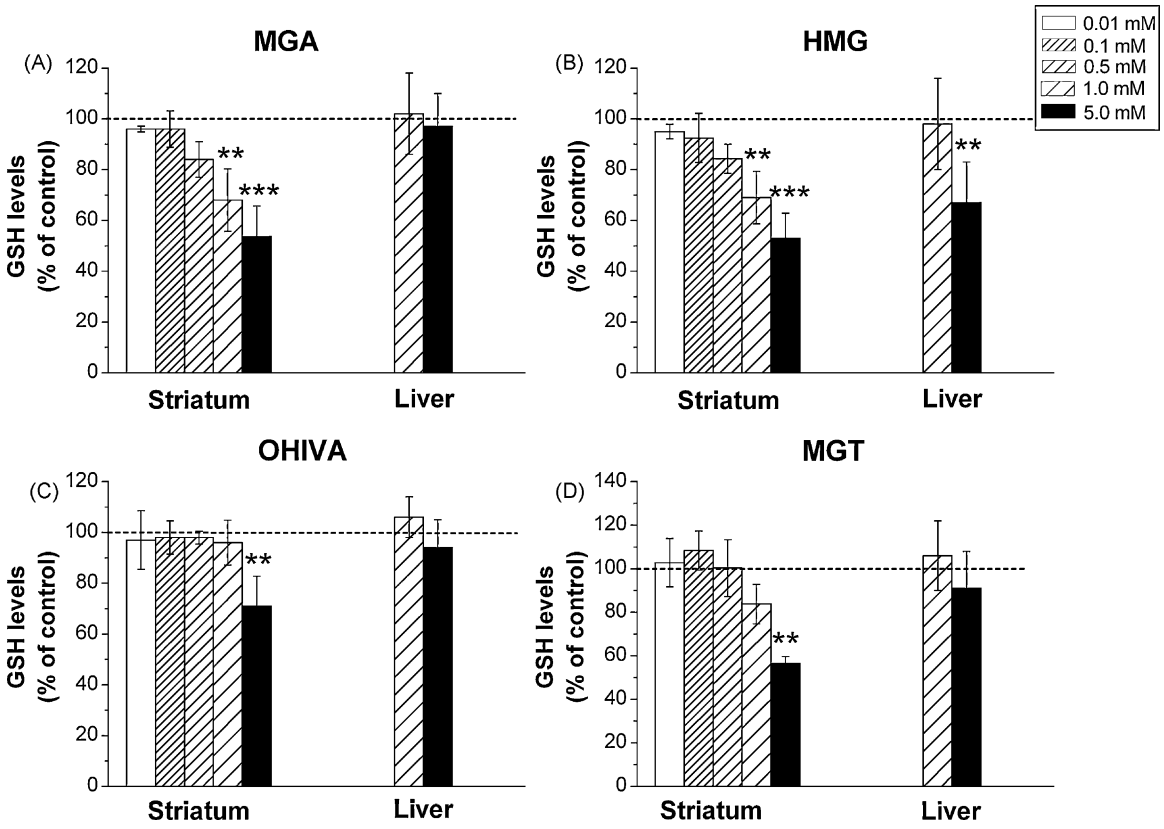


Fig. 2. Comparison between the *in vitro* effects of 3-methylglutaric acid (MGA; A), 3-hydroxy-3-methylglutaric acid (HMG; B), 3-hydroxyisovaleric acid (OHIVA; C) and 3-methylglutaconic acid (MGT; D) on glutathione (GSH) concentrations in striatum and liver of young rats. Data are represented as mean ± standard deviation of 3–7 animals for the striatum and seven for the liver, performed in triplicate and expressed as percentage of controls (100%) (controls [nmol/mg of protein]: A: striatum: 3.75 ± 0.49, liver: 5.41 ± 1.38; B: striatum: 4.07 ± 0.76, liver: 5.43 ± 1.26; C: striatum: 4.85 ± 0.62, liver: 5.43 ± 1.26; D: striatum: 4.87 ± 0.54, liver: 5.43 ± 1.26). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test).

damage induced by HMG in cerebral cortex (Leipnitz et al., 2008a). In this context, it should be emphasized that NAC is also an excellent radical scavenger itself, so that it is difficult at this time to establish whether NAC or its by-product GSH was more effective in trapping free radicals elicited by HMG (Leipnitz et al., 2008a). On the other hand, we cannot rule out that GSH decrease could be secondary to the increased reactive species generation provoked by HMG, MGT, MGA and OHIVA since GSH is the naturally most important free radical scavenger found in the brain.

We also observed that HMG, MGT and MGA reduced the sulfhydryl content (protein oxidation) in striatum, whereas OHIVA had no effect on this parameter. In contrast, protein oxidative damage was not observed in the liver preparations exposed to the same concentrations of these metabolites.

Therefore, based on the present findings and also on previous data reported on the oxidative effects of HMGA accumulating metabolites on the cerebral cortex (Leipnitz et al., 2008a,b), it may be presumed that brain structures are more vulnerable than liver to lipid and protein oxidative damage induced by these organic acids. This is supported by the fact that the brain is particularly sensitive to free radical attack due to its higher 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 (Halliwell and Gutteridge, 2007b). It should be also emphasized that the liver has a much higher regenerative capacity and adaptability to metabolic alterations regulating body homeostasis than the brain (Hallbergson et al., 2003; Tarla et al., 2006). Mitochondrial respiration rate was also shown to be higher in the brain compared to the liver, indicating that brain mitochondria potentially produce more ROS than peripheral tissues (Mori et al., 2007). It has been also revealed that the brain presents a higher peroxidation potential induced by ascorbate and Fe^{3+} relatively to the liver, as measured by TBA-RS levels and conjugated diene formation and greater susceptibility to the pro-oxidant effects of sulfur mustard (Pushpendran et al., 1994; Jafari, 2007).

All this strongly indicates that the brain is highly vulnerable to oxidative damage, compared to liver. It is therefore not surprising that brain is mainly injured in various inherited neurometabolic disorders, such as phenylketonuria (Sierra et al., 1998; Kienzle Hagen et al., 2002; Colome et al., 2003; Artuch et al., 2004; Sirtori et al., 2005; Sitta et al., 2006), maple syrup urine disease (Barschak et al., 2006), methylmalonic acidemia (Fontella et al., 2000; Ristoff and Larsson, 2002; Pettenuzzo et al., 2003), propionic acidemia (Pettenuzzo et al., 2002; Rigo et al., 2006) and glutaric acidemia type I (Kolker et al., 2001; de Oliveira Marques et al., 2003; Latini et al., 2005, 2007), in which oxidative stress plays an important role in their pathophysiology.

In conclusion, the present findings strengthen previous data showing that HMGA accumulating metabolites, especially HMG, induce lipid and protein oxidative damage and reduce the non-enzymatic antioxidant defenses in rat brain. More important, we provided evidence for a differential effect of the organic acids that accumulate in HMGA on oxidative attack in striatum and liver that may be tentatively attributed to specific characteristics of each tissue, such as oxygen consumption, metabolic activity rate, susceptibility to oxidants and many more factors directly or indirectly inducing free radical generation characteristic of the various organs of the body (Zaleska and Floyd, 1985; Beard et al., 1993; Kaushik and Kaur, 2003).

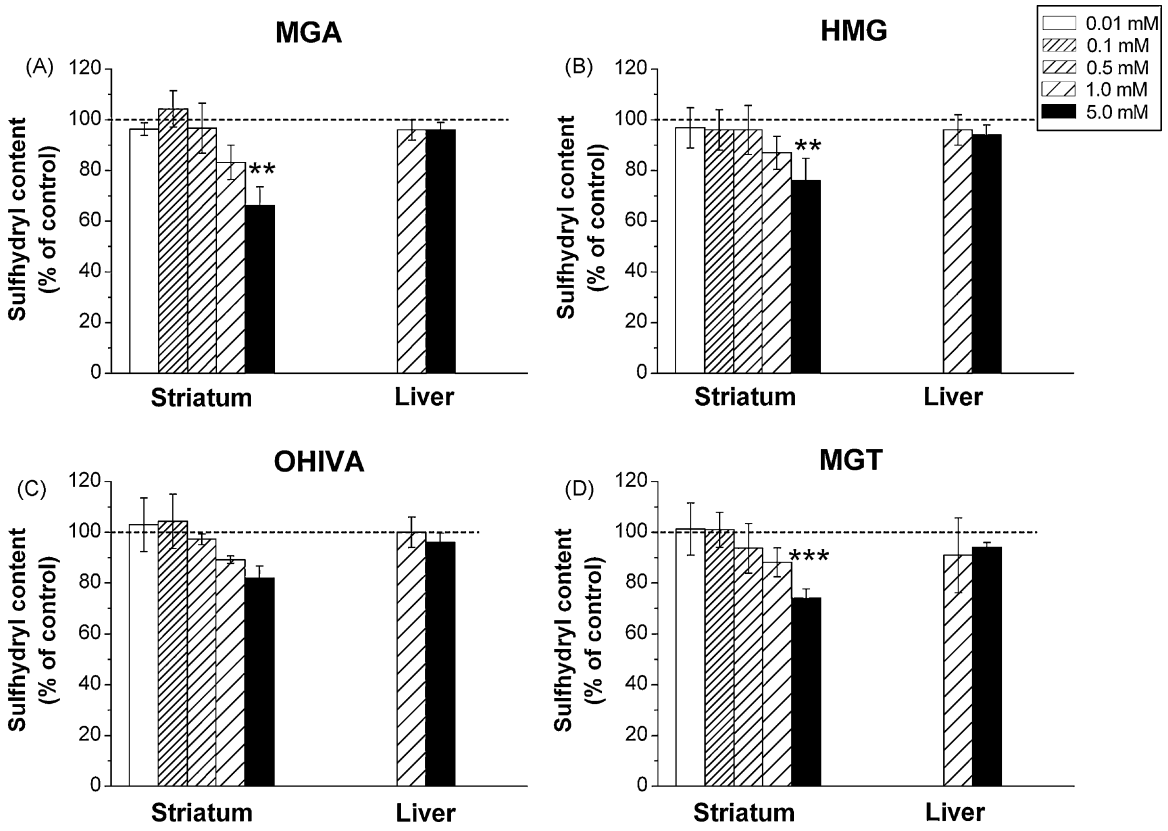


Fig. 3. Comparison between the *in vitro* effects of 3-methylglutaric acid (MGA; A), 3-hydroxy-3-methylglutaric acid (HMG; B), 3-hydroxyisovaleric acid (OHIVA; C) and 3-methylglutaconic acid (MGT; D) on reduced sulfhydryl content in striatum and liver of young rats. Data are represented as mean ± standard deviation of 3–7 animals for the striatum and 4–5 for the liver, performed in triplicate and expressed as percentage of controls (100%) (controls [nmol/mg of protein] – (A) striatum: 52.2 ± 6.9, liver: 71.2 ± 5.0; (B) striatum: 56.0 ± 6.0, liver: 66.8 ± 8.8; (C) striatum: 52.2 ± 6.9, liver: 71.2 ± 5.0; (D) striatum: 49.2 ± 5.4, liver: 71.2 ± 5.1). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test).

It is difficult to determine at the present the pathophysiological relevance of our data since to the best of our knowledge striatum and liver concentrations of the metabolites accumulating in patients affected by HMGA are not yet established. However, both striatum and cerebral cortex (Leipnitz et al., 2008a,b) revealed higher vulnerability to HMG, MGT, MGA and OHIVA than the liver, which corroborates with the fact that brain damage and neurological symptoms in HMGA-affected patients are more pronounced than hepatic dysfunction. Furthermore, mild liver damage with blood elevation of transaminases and ammonia mainly occurs during acute metabolic crises when the concentrations of these metabolites dramatically rise. In case the present findings are confirmed in tissues (for example, blood and skin fibroblasts) from HMGA patients, it is conceivable that oxidative stress may represent one important pathomechanism contributing to the tissue damage occurring in these patients. We have also to consider that hypoglycemia, which is commonly observed in HMGA during metabolic decompensation, may also induce reactive species generation in the brain through activation of neuronal glutamate receptors and increased calcium influx (Singh et al., 2004; Suh et al., 2007). In this context, it is conceivable that hypoglycemia may act synergistically with the accumulating metabolites eliciting reactive species production in HMGA. Therefore, the administration of antioxidants should be considered as an adjuvant therapy for patients affected by this disorder, especially during catabolic crises.

Acknowledgements

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.

References

Aksenov, M.Y., Markesbery, W.R., 2001. Change in thiol content and expression of glutathione redox system gene in the hippocampus and cerebellum in Alzheimer's disease. Neuroscience Letters 302, 141–145.

Artuch, R., Colome, C., Sierra, C., Brandi, N., Lambruschini, N., Campistol, J., Ugarte, D., Vilaseca, M.A., 2004. A longitudinal study of antioxidant status in phenylketonuric patients. Clinical Biochemistry 37, 198–203.

Barschak, A.G., Sitta, A., Deon, M., de Oliveira, M.H., Haeser, M., Dutra-Filho, C.S., Wajner, M., Vargas, C.R., 2006. Evidence that oxidative stress is increased in plasma from patients with maple syrup urine disease. Metabolic Brain Disease 21, 279–286.

Beard, J.L., Connor, J.R., Jones, B.C., 1993. Iron in the brain. Nutrition Reviews 51 (6), 157–170.

Behl, C., Moosmann, B., 2002. Oxidative nerve cell death in Alzheimer's disease and stroke: antioxidants as neuroprotective compounds. Biological Chemistry 383, 521–536.

Berg, D., Youdim, M.B., 2006. Role of iron in neurodegenerative disorders. Topics in Magnetic Resonance Imaging 17, 5–17.

Bogdanov, M.B., Andreassen, O.A., Dedeoglu, A., Ferrante, R.J., Beal, M.F., 2001. Increased oxidative damage to DNA in a transgenic mouse of Huntington's disease. Journal of Neurochemistry 79, 1246–1249.

Bonafe, L., Troxler, H., Kuster, T., Heizmann, C.W., Chamoles, N.A., Burlina, A.B., Blau, N., 2000. Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic aciduria. Molecular Genetics and Metabolism 69, 302–311.

Browne, R.W., Armstrong, D., 1998. Reduced glutathione and glutathione disulfide. Methods in Molecular Biology 108, 347–352.

Castoldi, A.F., Coccini, T., Ceccatelli, S., Manzo, L., 2001. Neurotoxicity and molecular effects of methylmercury. Brain Research Bulletin 55, 197–203.

Colome, C., Artuch, R., Vilaseca, M.A., Sierra, C., Brandi, N., Lambruschini, N., Cambra, F.J., Campistol, J., 2003. Lipophilic antioxidants in patients with phenylketonuria. The American Journal of Clinical Nutrition 77, 185–188.

de Oliveira Marques, F., Hagen, M.E., Pederzolli, C.D., Sgaravatti, A.M., Durigon, K., Testa, C.G., Wannmacher, C.M.D., Wyse, A.T.S., Wajner, M., Dutra-Filho, C.S., 2003. Glutaric acid induces oxidative stress in brain of young rats. Brain Research 964, 153–158.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods in Enzymology 186, 407–421.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., Lissi, E., 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Archives of Biochemistry and Biophysics 388 (2), 261–266.

Ferris, N.J., Tien, R.D., 1993. Cerebral MRI in 3-Hydroxy-3-methylglutaryl coenzyme A lyase deficiency: case report. Neuroradiology 35, 559–560.

Fontella, F.U., Pulrolnik, V., Gassen, E., Wannmacher, C.M., Klein, A.B., Wajner, M., Dutra-Filho, C.S., 2000. Propionic and L-methylmalonic acids induce oxidative stress in brain of young rats. Neuroreport 11, 541–544.

Funghini, S., Pasquini, E., Cappellini, M., Donati, M.A., 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. Molecular Genetics and Metabolism 73, 268–275.

Gibson, K.M., Breuer, J., Nyhan, W.L., 1988. 3-Hydroxy-3-methylglutarylcoenzyme A lyase deficiency: review of 18 reported patients. European Journal of Pediatrics 148, 180–186.

Gibson, K.M., Cassidy, S.B., Seaver, L.H., Wanders, R.J., Kennaway, N.G., Mitchell, G.A., Spark, R.P., 1994. Fatal cardiomyopathy associated with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. Journal of Inherited Metabolic Disease 17, 291–304.

Gordon, K., Riding, M., Camfild, P., Bawden, H., Ludman, M., Bagnell, P., 1994. CT and MR of 3-Hydroxy-3-methylglutaryl coenzyme A lyase deficiency. American Journal of Neuroradiology 15, 1474–1476.

Hallbergson, A.F., Gnatenco, C., Peterson, D.A., 2003. Neurogenesis and brain injury: managing a renewable resource for repair. The Journal of Clinical Investigation 112, 1128–1133.

Halliwell, B., Gutteridge, J.M.C., 2007a. Measurement of reactive species. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. 4th ed. Oxford University Press, Oxford, pp. 268–340.

Halliwell, B., Gutteridge, J.M.C., 2007b. Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. 4th ed. Oxford University Press, Oxford, pp. 268–340.

Jafari, M., 2007. Dose- and time-dependent effects pf sulfur mustard on antioxidant system in liver and brain of rat. Toxicology 231, 30–39.

Kaushik, S., Kaur, J., 2003. Chronic cold exposure affects the antioxidant defense system in various rat tissues. Clinica Chimica Acta 333, 69–77.

Kienzle Hagen, M.E., Pederzolli, C.D., Sgaravatti, A.M., Bridi, R., Wajner, M., Wannmacher, C.M.D., Wyse, A.T., Dutra-Filho, C.S., 2002. Experimental hyperphenylalaninemia provokes oxidative stress in rat brain. Biochimica et Biophysica Acta 1586, 344–352.

Kolker, S., Ahlemeyer, B., Krieglstein, J., Hoffmann, G.F., 2001. Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatric Research 50, 76–82.

Latini, A., Scussiato, K., Rosa, R.B., Leipnitz, G., Llesuy, S., Belló-Klein, A., Dutra-Filho, C.S., Wajner, M., 2003a. Induction of oxidative stress by L-2-hydroxyglutaric acid in rat brain. Journal of Neuroscience Research 74 (1), 103–110.

Latini, A., Scussiato, K., Rosa, R.B., Llesuy, S., Belló-Klein, A., Dutra-Filho, C.S., Wajner, M., 2003b. D-2-hydroxyglutaric acid induces oxidative stress in cerebral cortex of young rats. European Journal of Neuroscience 17 (10), 2017–2022.

Latini, A., Scussiato, K., Leipnitz, G., Dutra-Filho, C.S., Wajner, M., 2005. Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum. Journal of Inherited Metabolic Disease 28, 57–67.

Latini, A., Ferreira, G.C., Scussiato, K., Schuck, P.F., Solano, A.F., Dutra-Filho, C.S., Vargas, C.R., Wajner, M., 2007. Induction of oxidative stress by chronic and acute glutaric acid administration to rats. Cellular and Molecular Neurobiology 27 (4), 423–438.

Lee, C., Tsai, F.J., Wu, J.Y., Peng, C.T., Tsai, C.H., Hwu, W.L., Wang, T.R., Millington, D.S., 1999. 3-Hydroxy-3-methylglutaric aciduria presenting with Reye like syndrome: report of one case. Acta Paediatrica Taiwan 40, 445–447.

Leipnitz, G., Seminotti, B., Haubrich, J., Dalcin, M.B., 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-methylglutaric acid promotes lipid and protein oxidative damage and reduces the nonenzymatic antioxidant defenses in rat cerebral cortex. Journal of Neuroscience Research 86, 683–693.

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

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275.

Mancuso, M., Coppede, F., Migliore, L., Siciliano, G., Murri, L., 2006. Mitochondrial dysfunction, oxidative stress and neurodegeneration. Journal of Alzheimer's Disease 10, 59–73.

Mori, N., Yasutake, A., Hirayama, K., 2007. Comparative study of activities in reactive oxygen species production/defense system in mitochondria of rat brain and liver, and their susceptibility to methylmercury toxicity. Archives of Toxicology 81, 769–776.

Pettenuzzo, L.F., Schuck, P.F., Fontella, F., Wannmacher, C.M., Wyse, A.T., Dutra-Filho, C.S., Netto, C.A., Wajner, M., 2002. Ascorbic acid prevents cognitive deficits caused by chronic administration of propionic acid to rats in the water maze. Pharmacology, Biochemistry and Behavior 73, 623–629.

Pettenuzzo, L.F., Schuck, P.F., Wyse, A.T., Wannmacher, C.M., Dutra-Filho, C.S., Netto, C.A., Wajner, M., 2003. Ascorbic acid prevents water maze behavioral deficits caused by early postnatal methylmalonic acid administration in the rat. Brain Research 976, 234–242.

Pospisilova, E., Mrazova, L., Hrda, J., Martincova, O., Zeman, J., 2003. Biochemical and molecular analyzes in three patients with 3-hydroxy-3-methylglutaric aciduria. Journal of Inherited Metabolic Disease 26, 433–441.

Pushpendran, C.K., Subramanian, M., Devasagayam, T.P., 1994. Developmental changes in the peroxidation potential of rat brain homogenate and mitochondria. Mechanisms of Ageing and Development 73, 197–208.

Rigo, F.K., Pasquetti, L., Malfatti, C.R., Fighera, M.R., Coelho, R.C., Petri, C.Z., Mello, C.F., 2006. Propionic acid induces convulsions and protein carbonylation in rats. Neuroscience Letters 408, 151–154.

Ristoff, E., Larsson, A., 2002. Oxidative stress in inborn errors of metabolism: lessons from glutathione deficiency. Journal of Inherited Metabolic Disease 25, 223–226.

Sarafian, T.A., Bredesen, D.E., Verity, M.A., 1996. Cellular resistance to methylmercury. Neurotoxicology 17, 23–36.

Sgaravatti, A.M., Sgarbi, M.B., Testa, C.G., Durigon, K., Pederzolli, C.D., Prestes, C.C., Wyse, A.T.S., Wannmacher, C.M.D., Wajner, M., Dutra-Filho, C.S., 2007. γ-Hydroxybutyric acid induces oxidative stress in cerebral cortex of young rats. Neurochemistry International 50, 564–570.

Sierra, C., Vilaseca, M.A., Moyano, D., Brandi, N., Campistol, J., Lambruschini, N., Cambra, F.J., Deulofeu, R., Mira, A., 1998. Antioxidant status in hyperphenylalaninemia. Clinica Chimica Acta 276, 1–9.

Singh, P., Jain, A., Kaur, G., 2004. Impact of hypoglycemia and diabetes on CNS: Correlation of mitochondrial oxidative stress with DNA damage. Molecular and Cellular Biochemistry 260, 153–159.

Sirtori, L.R., Dutra-Filho, C.S., Fitarelli, D., Sitta, A., Haeser, A., Barschak, A.G., Wajner, M., Coelho, D.M., Llesuy, S., Bello-Klein, A., Giugliani, R., Deon, M., Vargas, C.R., 2005. Oxidative stress in patients with phenylketonuria. Biochimica et Biophysica Acta 1740, 68–73.

Sitta, A., Barschak, A.G., Deon, M., Terroso, T., Pires, R., Giugliani, R., Dutra-Filho, C.S., Wajner, M., Vargas, C.R., 2006. Investigation of oxidative stress parameters in treated phenylketonuric patients. Metabolic Brain Disease 21, 287–296.

Stoy, N., Mackay, G.M., Forrest, C.M., Christofides, J., Egerton, M., Stone, T.W., Darlington, L.G., 2005. Tryptophan metabolism and oxidative stress in patients with Huntington's disease. Journal of Neurochemistry 93, 611–623.

Suh, S.W., Gum, E.T., Hamby, A.M., Chan, P.H., Swanson, R.A., 2007. Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. The Journal of Clinical Investigation 117 (4), 910–918.

Sweetman, L., Williams, 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.

Tarla, M.R., Ramalho, F., Ramalho, L.N., Silva, T.C., Brandão, D.F., Ferreira, J., Silva, O.C., Zucoloto, S., 2006. Cellular aspects of liver regeneration. Acta Cirurgica Brasileira 21, 63–66.

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. American Journal of Neuroradiology 19, 378–382.

Vargas, C.R., Sitta, A., Schmitt, G., Ferreira, G.C., Cardoso, M.L., Coelho, D., Gibson, K.M., Wajner, M., 2008. Incidence of 3-hydroxy-3-methylglutaryl-coenzyme A lyase (HL) deficiency in Brazil, South America. Journal of Inherited Metabolic Disease 31 (1), 127–128.

Wysocki, S.J., Hahnel, R., 1986. 3-Hydroxy-3-methylglutaryl-coenzyme a lyase deficiency: a review. Journal of Inherited Metabolic Disease 9, 225–233.

Yalcinkaya, C., Dincer, A., Gündüz, E., Ficicioglu, C., Kocer, N., Aydin, A., 1999. MRI and MRS in HMG-CoA lyase deficiency. Pediatric Neurology 20, 375–380.

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

Zafeiriou, D.I., Vargiami, E., Mayapetek, E., Augoustidou-Savvopoulou, P., Mitchell, G.A., 2007. 3-Hydroxy-methylglutaryl Coenzyme A lyase deficiency with reversible white matter changes after treatment. Pediatric Neurology 37 (1), 47–50.

Zaleska, M.M., Floyd, R.A., 1985. Regional lipid peroxidation in rat brain *in vitro*: possible role of endogenous iron. Neurochemical Research 10, 397–410.

PARTE III

Discussão e Conclusões

III.1. DISCUSSÃO

A acidúria 3-hidroxi-3-metilglutárica (HMGA) é um erro inato do metabolismo que afeta a cetogênese e o catabolismo da leucina. As manifestações clínicas dos portadores dessa doença acometem principalmente o SNC, sendo que os pacientes afetados possuem lesões na substância branca, bem como envolvimento do trato corticoespinhal e dos núcleos caudato e denteado. Durante as crises, ocorrem também alterações no fígado dos pacientes, com hiperamonemia e aumento das transaminases aspartato e alanina aminotransferase, refletindo lesão hepática. A acidúria 3-metilglutacônica (MGTA), por sua vez, corresponde a um grupo heterogêneo de doenças caracterizado, predominantemente, por sintomas neurológicos.

A patogênese do dano cerebral presente nessas doenças é praticamente desconhecida, embora a hipoglicemia e também a hipocetonemia tenham sido propostos como fatores determinantes das lesões cerebrais na HMGA. Não se pode, no entanto, excluir a possibilidade de que os metabólitos ácidos acumulados nessas doenças possam ser neurotóxicos. O principal metabólito acumulado na HMGA é o ácido 3-hidroxi-3-metilglutárico (HMG), seguido dos ácidos 3-metilglutárico (MGA), 3-metilglutacônico (MGT) e 3-hidroxiisovalérico (OHIVA), ao passo que na MGTA há o acúmulo de MGT, MGA e OHIVA. Até onde sabemos, o único trabalho publicado demonstrou que o OHIVA não modifica as atividades dos complexos da cadeia respiratória, da creatina quinase, da Na+,K+-ATPase, bem como a produção de CO_2 em cérebro de ratos jovens, não alterando, portanto, o metabolismo energético cerebral (Ribeiro et al., 2007).

Assim, nos dois primeiros capítulos de nosso estudo investigamos os efeitos *in vitro* do HMG, do MGA, do MGT e do OHIVA sobre parâmetros de dano oxidativo a lipídios e proteínas e sobre as principais defesas antioxidantes não-enzimáticas e enzimáticas em córtex cerebral de ratos jovens, no intuito de contribuir para o esclarecimento dos mecanismos responsáveis pela patogênese da HMGA e da MGTA.

Inicialmente verificamos que o HMG, mesmo em baixas concentrações (0,25 mM), induziu peroxidação lipídica (aumento dos níveis de TBA-RS e quimioluminescência) em córtex cerebral. Também observamos que o MGT, o MGA e o OHIVA, em concentrações mais altas quando comparados com o HMG (a partir de 1 mM), aumentaram a medida de TBA-RS e a quimioluminescência em córtex cerebral. A medida de TBA-RS reflete a quantidade de malondialdeído (MDA) formado, que é um produto da oxidação de ácidos graxos poliinsaturados de lipídios complexos. O MDA é altamente tóxico, já que reage com proteínas e bases do DNA, causando alterações nos resíduos de aminoácidos e provocando mutações (Halliwell e Gutteridge, 2007b). Já a quimioluminescência avalia a emissão de luz decorrente de espécies formadas principalmente durante a peroxidação lipídica (Halliwell e Gutteridge, 2007b).

A seguir, estudamos o efeito de antioxidantes sobre o aumento de TBA-RS causado pelo HMG e pelo MGT, nas concentrações de 1 mM e 5 mM, respectivamente. Observamos que a melatonina (MEL, 200 µM) atenuou e que a N-acetilcisteína (NAC, 1 mM) preveniu totalmente o aumento de TBA-RS induzido pelo HMG, enquanto que o trolox (TRO, 1,5 µM), a glutationa (GSH, 100 µM), o N^{ω}-nitro-L-arginina metill éster (L-NAME; 500 µM), a combinação de

superóxido dismutase (SOD) e catalase (CAT) (2,5 U / mL de cada enzima) e a creatina (Cr, 3 mM) não foram capazes de alterar o efeito tóxico oxidativo do HMG. Por outro lado, altas concentrações de MEL (1,5 mM), de TRO (5 µM) e da combinação de SOD e CAT (10 mU / mL) preveniram totalmente a peroxidação lipídica induzida por esse ácido orgânico. Com relação ao MGT, também verificamos que apenas a MEL, em baixa concentração (200 µM), foi capaz de atenuar o aumento dos níveis de TBA-RS, enquanto que altas concentrações de MEL (1 mM), de TRO (5 µM) e da combinação de SOD e CAT (10 mU / mL) preveniram totalmente o efeito do MGT sobre a peroxidação lipídica. Tais resultados indicam o envolvimento de radicais livres nos efeitos do HMG e do MGT.

Verificamos também que o HMG aumentou significativamente a produção de ânion superóxido em partículas submitocondriais. Esses resultados, aliados ao fato de que o TRO atua principalmente contra radicais peroxila (Halliwell and Gutteridge, 2007a), a MEL contra os radicais hidroxila e peroxila (Reiter et al., 1997a, b, 2001; Maharaj et al., 2007) e a superóxido dismutase e a catalase agem sobre o ânion superóxido e o peróxido de hidrogênio, respectivamente, pode-se sugerir que essas espécies reativas estejam envolvidas na lipoperoxidação exercida pelo HMG. É importante ressaltar que as espécies reativas peróxido de hidrogênio e ânion superóxido não apresentam reatividade suficiente para iniciar o processo de peroxidação lipídica. Contudo, devemos considerar que essas espécies reativas geram o radical hidroxila através das reações de Fenton e Haber-Weiss, que é capaz de iniciar diretamente o processo de lipoperoxidação devido à sua alta reatividade (Halliwell e Gutteridge, 2007c).

Por outro lado, o MGT, o MGA e o OHIVA não alteraram a produção de ânion superóxido. Considerando que o MGT não alterou os níveis de ânion superóxido e que a MEL, o TRO e a combinação de SOD e CAT preveniram totalmente a peroxidação lipídica induzida por esse ácido, presume-se que os radicais hidroxila e peroxila, bem como o peróxido de hidrogênio estejam envolvidos nesse efeito. Esses resultados em seu conjunto indicam que o HMG, o MGT, o MGA e o OHIVA provocam um efeito pró-oxidante relevante sobre lipídeos de membrana em córtex cerebral provavelmente mediado pela geração de espécies reativas de oxigênio.

Convém enfatizar que o HMG e o MGT, nesta ordem, apresentaram os efeitos mais pronunciados dentre os ácidos orgânicos estudados. Finalmente, verificamos que o HMG não alterou os níveis de TBA-RS em mitocôndrias intactas e rompidas, sugerindo que o efeito desse metabólito sobre a lipoperoxidação *in vitro* depende de mecanismos citosólicos. Mais estudos são necessários, no entanto, para esclarecer quais mecanismos citosólicos poderiam estar envolvidos no dano lipídico provocado pelo HMG.

Por outro lado, observamos que o HMG, mesmo em baixa concentração (0,5 mM), aumentou a oxidação de sulfidrilas e a formação de carbonilas em córtex cerebral. O MGT e o MGA também induziram um aumento na formação de carbonilas e na oxidação de sulfidrilas, porém em concentrações mais altas (2,5 e 5 mM), ao passo que o OHIVA aumentou apenas a oxidação de sulfidrilas em córtex cerebral na concentração de 2,5 mM. Esses resultados indicam que todos os metabólitos analisados (HMG, MGT, MGA e OHIVA) induziram dano oxidativo protéico e que o HMG apresentou os efeitos mais

pronunciados, aumentando a formação de carbonilas e diminuindo o conteúdo de sulfidrilas.

É importante salientar que a maior parte dos equivalentes tiólicos nas proteínas é encontrada na forma reduzida e que tem sido proposto que esses grupamentos (PSH) são tão importantes quanto a GSH na manutenção do estado redox celular. Isso é possível porque, por um lado, os grupamentos sulfidrilas de proteínas constituem um “pool” maior que os grupamentos sulfidrilas da GSH e, por outro lado, porque durante a exposição a algum oxidante (radicais livres, por exemplo), os PSH podem ser reversivelmente oxidados formando pontes dissulfeto (PSSP) ou derivados glutationilados (PSSG), sendo reduzidos e regenerados após o restabelecimento do estado redox normal da célula (Hansen et al., 2009). Dessa forma, a oxidação de grupamentos sulfidrilas causada pelos ácidos orgânicos estudados representa tanto dano protéico quanto diminuição das defesas antioxidantes não-enzimáticas no córtex cerebral. Enfatize-se também que a oxidação dos grupos sulfidrila de determinadas proteínas (resíduos de cisteína específicos) geram dissulfetos, alterando o estado redox de proteínas e causando a sua inativação (Kuhn et al., 1999).

Além disso, devemos salientar que os grupos carbonilas (aldeídos e cetonas) são produzidos principalmente por oxidação de proteínas nas cadeias laterais (especialmente dos aminoácidos Pro, Arg, Lys, e Thr), por clivagem oxidativa, ou então por reações de redução de açúcares ou seus produtos de oxidação com resíduos protéicos de lisina (Dalle-Done et al., 2003). Por outro lado, o fato de que os radicais hidroxila, peroxila e superóxido e o peróxido de hidrogênio apresentarem reatividade suficiente para oxidar proteínas e formar

carbonilas (Dalle-Done et al., 2003; Halliwell e Gutteridge, 2007c), corrobora com nossos resultados descritos anteriormente indicando que essas espécies reativas estavam envolvidas na peroxidação lipídica induzida tanto pelo HMG quanto pelo MGT.

Nossos resultados também demonstraram que o HMG, o MGT, o MGA e o OHIVA não foram capazes de oxidar grupos tióis de uma solução comercial de GSH e de preparações de membranas mitocondriais purificadas. É, portanto, provável que a indução de dano protéico causado por esses ácidos orgânicos seja mediada via geração de espécies reativas de oxigênio e não por ataque oxidativo direto a grupamentos tiólicos.

No que diz respeito às defesas antioxidantes, o HMG diminuiu o potencial antioxidante total (TRAP), a reatividade antioxidante total (TAR) e o conteúdo de GSH, e isto está em consonância com os nossos achados demonstrando que o NAC, um precursor de GSH, preveniu a peroxidação lipídica induzida por HMG. Também verificamos que o provável radical envolvido nesse efeito foi o radical hidroxila. Tal conclusão baseou-se nos achados evidenciando que a MEL preveniu totalmente a diminuição dos níveis de GSH causados por HMG, enquanto os antioxidantes TRO e a combinação de SOD e CAT não interferiram sobre a diminuição das concentrações de GSH induzida por HMG. Esses achados que indicam um papel dos radicais hidroxila sobre a diminuição de GSH também reforçam os resultados observados com a peroxidação lipídica. Além disso, o MGA diminuiu o TRAP e as concentrações de GSH, enquanto que o MGT diminuiu as concentrações de GSH. O OHIVA não alterou nenhum dos parâmetros de defesas antioxidantes não-enzimáticas. A seguir, verificamos que a MEL (1 mM) e o TRO (10 μM), mas não a

combinação de SOD e CAT, preveniram totalmente a diminuição dos níveis de GSH causada pelo MGA, indicando um papel dos radicais hidroxila e peroxila nesse efeito. O TRAP e a TAR medem, respectivamente, a quantidade e a qualidade dos antioxidantes presentes na amostra. Já a GSH, por sua vez, é um importante antioxidante cerebral, sendo encontrado em concentrações na faixa de milimolar neste tecido. Considerando que o TRAP, a TAR e as concentrações de GSH são utilizados para avaliar a capacidade antioxidante não-enzimática dos tecidos em prevenir o dano associado aos processos gerados por radicais livres, pode-se presumir que as defesas antioxidantes não-enzimáticas do córtex cerebral de ratos foram comprometidas pelo HMG, pelo MGA e pelo MGT (Halliwell e Gutteridge, 2007a), sendo o HMG o metabólito com maior efeito sobre as defesas antioxidantes não-enzimáticas no cérebro. Observamos ainda para os experimentos realizados com HMG uma correlação inversa entre os valores de TBA-RS e das concentrações de GSH, indicando que a diminuição das defesas antioxidantes não-enzimáticas cerebrais e a indução de dano oxidativo causados pelo HMG foram presumivelmente devidas ao aumento da geração de espécies reativas por esse ácido orgânico.

Finalmente, nossos resultados também demonstraram que o HMG, o MGA, MGT e OHIVA não alteraram *in vitro* a atividade das enzimas antioxidantes SOD, CAT e glutationa peroxidase (GPx) em córtex cerebral (resultados não publicados). Tais resultados sugerem que esses metabólitos não são capazes de interagir diretamente ou afetar a atividade dessas enzimas indiretamente através da formação de espécies ativas *in vitro* nas concentrações utilizadas.

Uma vez que o estresse oxidativo resulta de um desequilíbrio entre as defesas antioxidantes e as espécies reativas geradas em um tecido, nossos presentes resultados fortemente indicam que os metabólitos acumulados na HMGA e na MGTA induzem estresse oxidativo em córtex cerebral de ratos jovens, uma condição deletéria à célula (Halliwell e Gutteridge, 2007a). Neste ponto, deveria ser enfatizado que o cérebro possui poucas defesas antioxidantes quando comparado com outros tecidos (Halliwell e Gutteridge, 1996), o que faz com que este tecido seja mais vulnerável ao aumento da produção de espécies reativas. De fato, o estresse oxidativo tem sido implicado na fisiopatologia de várias doenças neurodegenerativas comuns, tais como as doenças de Parkinson e de Alzheimer, bem como em epilepsia e na desmielinização (Halliwell e Gutteridge, 1996; Perez-Severiano et al., 2000; Bogdanov et al., 2001; Méndez-Álvarez et al., 2001; Karelson et al., 2001; Behl e Moosmann, 2002; Stoy et al., 2005; Berg e Youdim, 2006; Mancuso et al., 2006). Além disso, convém enfatizar que nossos presentes resultados não foram devidos a um efeito secundário dos metabólitos estimulando receptores de NMDA com conseqüente entrada de cálcio e produção de espécies reativas, uma vez que usamos frações celulares (sobrenadante de córtex cerebral) em que este mecanismo não poderia ocorrer. Estudos utilizando preparações com células íntegras devem, no entanto, ser realizados para esclarecer se os ácidos orgânicos acumulados na HMGA e MGTA são capazes de estimular receptores glutamatérgicos.

O próximo passo de nossa investigação foi o de comparar a suscetibilidade de tecidos cerebrais e periféricos ao dano oxidativo provocado pelos ácidos HMG, MGA, MGT e OHIVA, uma vez que os portadores da HMGA

apresentam atrofia cortical, bem como lesões nos núcleos caudato e denteado, além de hepatomegalia com hiperamonemia moderada durante as crises de descompensação metabólica. Investigamos, portanto, os efeitos *in vitro* do HMG, do MGA, do MGT e do OHIVA sobre os níveis de TBA-RS (peroxidação lipídica), conteúdo de sulfidrilas (dano protéico) e concentrações de GSH (defesas antioxidantes não-enzimáticas) em estriado (tecido central) e fígado (tecido periférico) de ratos jovens.

Verificamos que o HMG, dentre os quatro ácidos estudados, apresentou o maior efeito sobre a indução de peroxidação lipídica (aumento dos níveis de TBA-RS) no estriado e foi o único metabólito capaz de induzir lipoperoxidação no fígado. O MGA, o MGT e o OHIVA também aumentaram significativamente a medida de TBA-RS no estriado, mas não no fígado.

Também observamos que todos ácidos orgânicos estudados diminuíram significativamente as concentrações de GSH em estriado, sendo que o HMG foi o que teve efeito mais pronunciado. Além disso, apenas o HMG diminuiu os níveis desse importante antioxidante no fígado. É possível que a redução dos níveis de GSH esteja relacionada, ao menos em parte, com a indução de peroxidação lipídica causada pelo HMG, visto que a GSH é um importante antioxidante necessário à eliminação de hidroperóxidos lipídicos e de radicais peroxila, que são produzidos no processo de lipoperoxidação. Outras observações do presente estudo que suportam essa hipótese são os resultados demonstrando que o NAC, um clássico precursor de GSH, preveniu o aumento dos níveis de TBA-RS causado pelo HMG em córtex cerebral. Outra possibilidade é que a diminuição das concentrações de GSH pode ter sido

devida ao aumento na produção de espécies reativas causado pelo HMG, MGT, MGA e OHIVA.

Nossos estudos também mostraram que o HMG, o MGA e o MGT aumentaram a oxidação de grupamentos sulfidrila em estriado, ao passo que nenhum dos metabólitos alterou esse parâmetro no fígado.

Portanto, considerando os resultados encontrados para o córtex cerebral (capítulos I e II) e também para o estriado (capítulo III) de que os metabólitos acumulados na HMGA e na MGTA provocam um maior dano oxidativo lipídico e protéico, relativamente ao dano causado ao fígado, concluímos que as estruturas cerebrais são mais vulneráveis ao estresse oxidativo induzido por esses metabólitos. Nesse contexto, é importante ressaltar que o cérebro é altamente vulnerável ao dano oxidativo, pois possui uma alta taxa de metabolismo oxidativo que ocorre concomitantemente com a produção de espécies reativas, baixo conteúdo de defesas antioxidantes quando comparado a outros tecidos, grande quantidade de neurotransmissores auto-oxidáveis, ferro e ácidos graxos poliinsaturados, que favorecem o processo de peroxidação lipídica (Halliwell e Gutteridge, 2007b). Além disso, outros estudos demonstraram que o cérebro apresenta maior suscetibilidade à lipoperoxidação induzida por ácido ascórbico e Fe^{3+} quando comparado ao fígado (Pushpendran et al., 1994) e que a taxa de respiração mitocondrial é maior no cérebro, indicando que frações mitocondriais obtidas de cérebro produzem mais espécies reativas que tecidos periféricos (Mori et al., 2007).

As manifestações clínicas apresentadas por pacientes com HMGA são predominantemente neurológicas, ao passo que alguns afetados apresentam disfunção hepática com hiperamonemia e elevação de transaminases,

refletindo lesão hepática, apenas em situações de crise metabólica aguda, quando ocorrem elevações dramáticas nas concentrações dos metabólitos acumulados. Assim, é possível que a maior vulnerabilidade apresentada pelo cérebro (córtex cerebral e estriado) ao dano oxidativo causado por HMG, MGA, MGT e OHIVA quando comparado com o fígado, poderia estar relacionada aos sintomas apresentados por esses pacientes.

Não podemos estabelecer a importância fisiopatológica dos resultados aqui apresentados, já que as concentrações cerebrais dos ácidos acumulados na HMGA e na MGTA não são conhecidas. Contudo, é importante salientar que os efeitos exercidos pelo HMG ocorreram com concentrações relativamente baixas (0,25 mM e concentrações maiores). Por outro lado, os sintomas neurológicos severos encontrados nos pacientes se agravam durante crises de descompensação metabólica, nas quais ocorrem elevações substanciais nas concentrações desses metabólitos. Além disso, é possível que os ácidos orgânicos testados, por serem ácidos dicarboxílicos, também se acumulem nas células neurais, por dificuldade no efluxo das mesmas, como ocorre com os ácidos glutárico e 3-hidroxiglutárico, que são semelhantes estruturalmente (Hoffmann et al., 1993, 1994; Sauer et al., 2006; Stellmer et al., 2007).

Concluindo, relatamos pela primeira vez que os metabólitos HMG, MGA, MGT e OHIVA, que se acumulam na HMGA e na MGTA, induzem estresse oxidativo em tecidos cerebrais através de um mecanismo independente dos receptores NMDA. Também verificamos que o HMG induziu estresse oxidativo no fígado, mas em grau menor. No caso de ser confirmado que esses metabólitos induzem a produção de radicais livres *in vivo* no cérebro, poderia presumir-se que a produção elevada destes radicais altamente reativos no

cérebro seria potencialmente deletéria ao SNC. Nesse caso, o uso de agentes antioxidantes poderia representar uma nova abordagem terapêutica, junto com outras medidas, no tratamento dos pacientes afetados por esses distúrbios genéticos.

III.2. CONCLUSÕES

- Os ácidos 3-hidroxi-3-metilglutárico (HMG), 3-metilglutárico (MGA), 3-metilglutacônico (MGT) e 3-hidroxiisovalérico (OHIVA), acumulados nas acidúrias 3-hidroxi-3-metilglutárica e 3-metilglutacônica, induziram lipoperoxidação (dano oxidativo lipídico determinado através do aumento da medida das substâncias reativas ao ácido tiobarbitúrico e quimioluminescência) em córtex cerebral e estriado de ratos jovens, enquanto no fígado apenas o HMG provocou esse efeito.
- A lipoperoxidação induzida pelo HMG foi prevenida pelos antioxidantes melatonina (MEL), N-acetilcisteína (NAC), trolox (TRO) e a combinação de superóxido dismutase (SOD) e catalase (CAT), indicando o envolvimento dos radicais hidroxila, peroxila e ânion superóxido, bem como do peróxido de hidrogênio no efeito observado.
- No caso do MGT, o dano oxidativo lipídico foi prevenido ou atenuado pelos antioxidantes MEL, TRO e a combinação de SOD e CAT, indicando uma ação dos radicais hidroxila e peroxila, bem como do peróxido de hidrogênio no efeito observado.
- O ácido HMG aumentou significativamente a produção de ânion superóxido em partículas submitocondriais de córtex cerebral de ratos jovens, enquanto os ácidos MGA, MGT e OHIVA não alteraram a formação desse radical livre.
- Os ácidos HMG, MGA e MGT provocaram dano oxidativo protéico, evidenciado pelo aumento da formação de carbonilas e da oxidação de grupamentos sulfidrila em córtex cerebral de ratos jovens.

- Os ácidos HMG, MGA, MGT e OHIVA provocaram dano oxidativo protéico em córtex cerebral de ratos jovens, já que aumentaram significativamente a oxidação de grupamentos sulfidrilas e a formação de carbonilas, ao passo que o HMG, o MGA e o MGT também induziram dano protéico no estriado.
- Os ácidos HMG e MGA diminuíram significativamente o potencial antioxidante total (TRAP), enquanto o ácido HMG diminuiu significativamente também a reatividade antioxidante total (TAR) em córtex cerebral de ratos jovens.
- Os ácidos HMG, MGA e MGT diminuíram significativamente as concentrações de GSH em córtex cerebral de ratos jovens, ao passo que o HMG, o MGA, o MGT e o OHIVA diminuíram esse parâmetro no estriado. Por outro lado, apenas o HMG diminuiu as concentrações desse antioxidante no fígado.
- A diminuição das concentrações de GSH causada pelo HMG em córtex cerebral de ratos jovens foi prevenida pelo antioxidante MEL, indicando que os radicais hidroxila estão envolvidos nesse efeito.
- Já no efeito causado pelo MGA, os antioxidantes MEL e TRO preveniram a diminuição das concentrações de GSH em córtex cerebral de ratos jovens, indicando que radicais hidroxila e peroxila têm participação nesse efeito.
- Os ácidos HMG, MGA, MGT e OHIVA não alteraram as atividades das enzimas antioxidantes SOD, CAT e glutationa peroxidase (GPx) em córtex cerebral de ratos jovens.

- Os ácidos HMG, MGA, MGT e OHIVA não alteraram a oxidação dos grupamentos tióis em uma solução comercial de GSH e em preparações de membranas mitocondriais purificadas na ausência de sobrenadante, sugerindo que não atacam diretamente esses grupamentos.
- O dano oxidativo importante provocado pelos metabólitos acumulados na HMGA e na MGTA obtido em tecidos centrais (córtex cerebral e estriado) e pouco evidenciado no fígado sugerem que o cérebro é mais suscetível ao ataque de radicais livres, o que poderia explicar, ao menos em parte, as manifestações clínicas neurológicas predominantes apresentadas por pacientes afetados por HMGA e MGTA.

III.3. PERSPECTIVAS

- Avaliar os efeitos *in vitro* dos ácidos 3-hidroxi-3-metilglutárico (HMG), 3-metilglutárico (MGA), 3-metilglutacônico (MGT) e 3-hidroxiisovalérico (OHIVA) sobre processo de oxidações biológicas em músculo cardíaco e cerebelo de ratos jovens, bem como em partículas submitocondriais de diferentes estruturas cerebrais de ratos jovens.
- Avaliar os efeitos *in vitro* dos ácidos HMG, MGA, MGT e OHIVA sobre parâmetros de metabolismo energético e função mitocondrial em diferentes estruturas e tecidos de ratos jovens.
- Avaliar os efeitos *ex vivo* de injeções intraperitoneais (IP) e intracerebrais dos ácidos HMG, MGA, MGT e OHIVA sobre processos de oxidações biológicas (estresse oxidativo) e metabolismo energético em várias estruturas cerebrais (córtex, estriado, hipocampo e cerebelo) de ratos na presença ou ausência de agentes permeabilizantes da barreira hematoencefálica.
- Avaliar os efeitos *ex vivo* de injeções IP e intracerebrais dos ácidos HMG, MGA, MGT e OHIVA sobre processos de oxidações biológicas (estresse oxidativo) em várias estruturas cerebrais (córtex, estriado, hipocampo e cerebelo) de ratos na presença ou ausência de neuroinflamação induzida pela administração IP (inflamação sistêmica) ou intracerebroventricular (ICV) (neuroinflamação) de lipopolissacarídeo.

REFERÊNCIAS

Aksenov, M.Y., Markesbery, W.R. Change in thiol content and expression of glutathione redox system gene in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 302:141-145, 2001.

Alexi, T., Borlongan, C.V., Faull, R.L., Williams, C.E., Clark, R.G., Gluckman, P.D., Hughes, P.E. Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. Prog Neurobiol, 409-70, 2000.

Al-Sayed, M., Imtiaz, F., Alsmadi, O.A., Rashed, M.S., Meyer, B.F. Mutations underlying 3-hydroxy-3-methylglutaril CoA lyase deficiency in the Saudi population. BMC Med Genet 7: 86, 2006.

Anikster, Y., Kleta, K., Shaag, A., Ghal, W.A., Elpeleg, O. Type III 3-methylglutaconic aciduria (optic atrophy plus syndrome, or Costeff optic atrophy syndrome): identification of OPA3 gene and its founder mutation in Iraqi Jews. AM J Hum Genet 69: 1218-1224, 2005.

Arbelaez, A., Castillo, M., Stone, J. MRI in 3-methylglutaconic aciduria type 1. Neuroradiology 41: 941-942, 1999.

Arn, P., Funanage, V.L. 3-Methylglutaconic aciduria disorders: The clinical spectrum increases. J Pediatr Hematol Oncol 28(2): 62-63, 2006.

Baric, I., Furnic, K., Hoffmann, G.F. Inborn errors of metabolism at the turn of the millennium. Croat Med J 42:379-383, 2001.

Barschak, A.G., Marchesan, C., Sitta, A., Deon, M., Giugliani, R., Wajner, M., Vargas, C.R. Maple syrup urine disease in treated patients: biochemical and oxidative stress profiles. Clin Biochem 41: 317-324, 2008a.

Barschak, A.G., Sitta, A., Deon, M., Barden, A.T., Dutra-Filho, C.S., Wajner, M., Vargas, C.R. Oxidative stress in plasma from maple syrup urine disease patients during treatment. Metab Brain Dis 23: 71-80, 2008b.

Barth, P.G., Scholte, H.R., Berden, J.A. An X-linked mitochondrial disease affecting cardiac muscle, skeletal muscle and neutrophil leukocytes. J Neurol Sci 62: 327-355, 1983.

Barth, P.G., Valianpour, F., Bowen, V.M. Lam, J., Duran, M., Vaz, F.M., Wanders, R.J. X-linked cardioskeletal myopathy and neutropenia (Barth syndrome): an update. Am J Med Genet A 126: 349-354, 2004.

Behl, C., Moosmann, B. Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. Free Radic Biol Med 33: 182-191, 2002.

Berg, D., Youdim, M.B. Role of iron in neurodegenerative disorders. Top Magn Reson Imaging 17: 5-17, 2006.

Bergendi, L., Benes, L., Duracková, Z., Ferencik, M. Chemistry, physiology and pathology of free radicals. Life Sci 65: 1865-1874, 1999.

Besley, G.T.N., Lendon, M., Broadhead, D.M., Till, J., Heptinstall, L.E., Phillips, B. Mitochondrial complex deficiencies in a male with cardiomyopathy and 3-methylglutaconic aciduria. J Inherit Metab Dis 18: 221-223, 1995.

Bickel, H. Early diagnosis and treatment of inborn errors of metabolism. Enzyme 38:14-26, 1987.

Bione, S., D'Adamo, P., Maestrini, E., Gedeon, A.K., Bolhuis, P.A., Toniolo, D. A novel X-linked, G4.5 is responsible for Barth syndrome. Nat Genet 12: 385-389, 1996.

Bogdanov, M.B., Andreassen, O.A., Dedeoglu, A., Ferrante, R.J., Beal, M.F. Increased oxidative damage to DNA in a transgenic mouse of Huntington's disease. J Neurochem 79: 1246-1249, 2001.

Bolhuis, P.A., Page-Christiaens, G.C. The advisory report "Neonatal screening" from the Health Council of The Netherlands. Ned Tijdschr Geneeskd 149(51): 2857-2860, 2005.

Bonafé, L., Troxler, H., Kuster, T., Heizmann, C.W., Chamoles, N.A., Burlina, A.B., Blau, N. Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic acidurias. Mol Genet Metab 69: 302-311, 2000.

Boveris, A. Biochemistry of free radicals: from electrons to tissues. Medicina (B. Aires) 58: 350-356, 1998.

Browne, R.W., Armostrong, D. Reduced glutathione and glutathione disulfide. Methods Mol Biol 108: 347-352, 1998.

Chalmers, R.A., Purkiss, P., Watts, R.W., Lawson, A.M. Screening for organic acidurias and amino acidopathies in newborns and children. J Inherit Metab Dis 3: 27-43, 1980.

Chalmers, R.A., Lawson, A.M. Organic acids in man. Analytical chemistry, biochemistry and diagnosis of the organic acidurias. London, Chapman & Hall. 221-229, 1982.

Christodoulou, J., McInes, R.R, Jay, V., Wilson, G., Becker, L.E., Lehotay, D.C., Platt, B.A., Bridge, P.J., Robinson, B.H., Clarke, J.T. Barth syndrome: clinical observations and genetic linkage studies. Am J Med Genet 50: 255-264, 1994.

Da Cruz, S., Xenarios, I., Langridge, J., Vilbois, F., Parone, P.A., Martinou, J.C. Proteomic analysis of the mouse liver mitochondrial inner membrane. J Biol Chem 278: 41566-41571, 2003.

Dalle-Done, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 329: 23-38, 2003.

Dasouki, M., Buchanan, D., Mercer, N., Gibson, K.M., Thoene, J. 3-Hydroxy-3-methylglutaric aciduria: response to carnitine therapy and fat and leucine restriction. J Inherit Metab Dis 10(2):142-146, 1987.

Davey, K.M., Parboosingh, J.S., McLeod, D.R., Chan, A., Casey, R., Ferreira, P., Snyder, F.F., Bridge, P.J., Bernier, F.P. Mutation of DNAJC19, a human homologue of yeast inner mitochondrial membrane co-chaperones, causes DCMA syndrome, a novel autosomal recessive Barth syndrome-like condition. J Med Genet 43: 385-393, 2006.

Davies, V.J., Powell, K.A., White, K.E., Yip, W., Hogan, V., Hollins, A.J., Davies, J.R., Piechota, M., Browstein, D.G., Moat, S.J., Nichols, P.P., Wride, M.A., Boulton, M.E., Votruba, M. A minissense mutation in the murine *OPA3* gene models human Costeff syndrome. Brain 131: 368-380, 2008.

De Kremer, R.D., Paschini-Capra, A., Bacman, S., Argaraña, C., Civallero, G., Kelley, R.I., Guelbert, N., Latini, A., Halac, I.N., Giner-Ayala, A., Johnston, J., Proujansky, R., Gonzalez, I., Depetris-Boldini, C., Oller-Ramírez, A., Angaroni, C., Theaux, R.A., Hliba, E., Juaneda, E. Barth's syndrome-like disorder: A new phenotype with a maternally inherited A3243G substitution of mitochondrial DNA (MELAS mutation). Am J Med Genet 99: 83-93, 2001.

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

Deon, M., Sitta, A., Barschak, A.G., Coelho, D.M., Pigatto, M., Schmitt, G.O., Jardim, L.B., Giugliani, R., Wajner, M., Vargas, C.R. Induction of lipid peroxidation and decrease of antioxidant defenses in symptomatic and asymptomatic patients with X-linked adrenoleukodystrophy. Int J Dev Neurosci 25: 441-4, 2007.

Deon, M., Garcia, M.P., Sitta, A., Barschak, A.G., Coelho, D.M., Schimit, G.O., Pigatto, M., Jardim, L.B., Wajner, M., Giugliani, R., Vargas, C.R. Hexacosanoic and docosanoic acids plasma levels in patients with cerebral childhood and asymptomatic X-linked adrenoleukodystrophy: Lorenzo's oil effect. Metab Brain Dis 23: 43-49, 2008.

Faull, K.F., Bolton, P.D., Halpern, B., Hammond, J., Danks, D.M. The urinary organic acid profile associated with 3-hydroxy-3-methylglutaric aciduria. Clin Chim Acta 73: 553-559, 1976a.

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

Feksa, L.R., Latini, A., Rech, V.C., Feksa, P.B., Koch, G.D., Amaral, M.F., Leipnitz, G., Dutra-Filho, C.S., Wajner, M., Wannmacher, C.M. Tryptophan administration induces oxidative stress in brain cortex of rats. Metab Brain Dis 23: 221-233, 2008.

Flecknell, P.A, Cruz, I.J., Liles, J.H., Whelan, G. Induction of anaesthesia with halotone and isofluratone in the rabbit: a comparasion os the use of a face-mask or an anaesthetic chamber. Lab Anim 30(1): 67-74, 1996.

Funghini, S., Pasquini, E., Cappellini, M., Donati, M.A., Morrone, A., Fonda, C., Zammarchi, E. 3-Hydroxy-3-methylglutaric aciduria in an Italian patient is caused by a new nonsense mutation in the HMGDL gene. Mol Genet Metab 73:268–275, 2001.

Gibson, K.M., Bennett, M.J., Mize, C.E., Jakobs, C., Rotig, A., Munnich, A., Lichter-Konecki, U., Trefz, F.K. 3-Methylglutaconic aciduria associated with Pearson syndrome and respiratory chain defects. J Pediatr 121: 940-942, 1992.

Gibson, K.M., Wappner, R.S., Jooste, S., Erasmus, E., Mienie, L.J., Gerlo, E., Desprechins, B., De Meirleir, L. Variable clinical presentation in three patients with 3-methylglutaconyl-coenzyme A hydratase deficiency. J Inherit Metab Dis 21:631-638, 1998.

Gu, M., Owen, A.D., Toffa, S.E., Cooper, J.M., Dexter, D.T., Jenner, P., Marsden, C.D., Schapira, A.H. Mitochondrial function, GSH and iron in neurodegeneration and Lewy body diseases. J Neurol Sci 158: 24-29, 1998.

Gunay-Aygun, M. IEM digest. Mol Genet Metab 84: 1-3, 2005.

Halliwell, B., Gutteridge, J.M.C. Oxygen radicals and nervous system. Trends Neurosci 8: 22-26, 1996.

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

Halliwell, B., Whiteman, M. Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142: 231-255, 2004.

Halliwell, B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141:312-22, 2006.

Halliwell, B., Gutteridge, J.M.C. Measurement of reactive species. In: Halliwell, B., Gutteridge, J.M.C, editores. Free radicals in biology and medicine. Oxford: Oxford University Press, p. 268–340, 2007a.

Halliwell, B., Gutteridge, J.M.C. Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. In: Halliwell, B., Gutteridge, J.M.C., editores. Free radicals in biology and medicine. Oxford: Oxford University Press, p. 187–267, 2007b.

Halliwell, B., Gutteridge, J.M.C. Antioxidant defences. In: Halliwell, B., Gutteridge, J.M.C., editores. Free radicals in biology and medicine. Oxford: Oxford University Press, p. 79–185, 2007c.

Hansen, R.E., Roth, D., Winther, J.R. Quantifying the global cellular thiol-disulfide status. PNAS 106(2): 422-427, 2009.

Hoffmann, G.F., Meier-Augenstein, W., Stockler, S., Surtees, R., Rating, D., Nyhan, W.L. Physiology and pathophysiology of organic acids in cerebrospinal fluid. J Inherit Metab Dis 16: 648-669, 1993.

Hoffmann, G.F., Gibson, K.M., Trefz, F.K., Nyhan, W.L., Bremer, H.J., Rating, D. Neurological manifestations of organic acid disorders. Eur J Pediatr 153: S94-S100, 1994.

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

Ibel, H., Endres, W., Hadorn, H.B., Deufel, T., Paetzke, I., Duran, M., Kennaway, N.G., Gibson, K.M. Multiple respiratory chain abnormalities associated with hypertrophic cardiomyopathy and 3-methyl-glutaconic aciduria. Eur J Pediatr 152: 665-670, 1993.

Ijlst, L., Loupatty, F.J., Ruiter, J.P.N., Duran, M., Lehnert, W., Wanders, R.J.A. 3-Methylglutaconic aciduria type I is caused by mutations in *AUH*. Am J Hum Genet 71: 1463-1466, 2002.

Janetzky, B., Hauck, S., Youdim, M.B., Riederer, P., Jellinger, K., Pantucek, F., Zöchling, R., Boissl, K.W., Reichmann, H. Unaltered aconitase activity, but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. Neurosci Lett 169: 126-128, 1994.

Karelson, E., Bogdanovic, N., Garlind, A., Winblad, B., Zilmer, K., Kullisaar, T., Vihalemm, T., Kairane, C., Zilmer, M. The cerebrocortical areas in normal brain aging and in the Alzheimer's disease: noticeable differences in the lipid peroxidation level and in antioxidant defense. Neurochem Res 26: 353-361, 2001.

Kessler, A., Biasibetti, M., da Silva Melo, D.A., Wajner, M., Dutra-Filho, C.S., Wyse, A.T.S., Wannmacher, C.M.D. Antioxidant effect of cysteamine in brain cortex of young rats. Neurochem Res 33: 737-744, 2008.

Kuhn, D.M., Aretha, C.W., Geddes, T.J. Peroxynitrite Inactivation of Tyrosine Hydroxylase: Mediation by Sulfhydryl Oxidation, not Tyrosine Nitration. J Neurosci 19: 10289-10294, 1999.

Latini, A., Scussiato, K., Leipnitz, G., Gibson, K.M., Wajner, M. Evidence for oxidative stress in tissues derived from succinate semialdehyde dehydrogenase-deficient mice. J Inherit Metab Dis 30: 800-810, 2007.

Lovell, M.A., Xie, C., Markesbery, W.R. Decreased base excision repair and increased helicase activity in Alzheimer's disease brain. Brain Res 855: 116-123, 2000.

Maharaj, D.S., Glass, B.D., Daya, S. Melatonin: new places in therapy. Biosci Reports 27: 299-320, 2007.

Mancuso, M., Coppede, F., Migliore, L., Siciliano, G., Murri, L. Mitochondrial dysfunction, oxidative stress and neurodegeneration. J Alzheimers Dis 10: 59-73, 2006.

Markesbery, W.R., Carney, J.M. Oxidative alterations in Alzheimer's disease. Brain Pathol 9: 133-146, 1999.

Maxwell, S.R. Prospects for the use of antioxidant therapies. Drugs 49: 345-361, 1995.

Mayatepek, E., Hoffmann, G.F., Baumgartner, R., Schulze, A., Jakobs, C., Trefz, F.K., Bremer, H.J. Atypical vitamin B12-uresponsive methylmalonic aciduria in sibship with severe progressive encephalomyopathy: a new genetic disease? Eur J Pediatr 155: 398-403, 1996.

McKenzie, M., Lazarou, M., Thorburn, D.R., Ryan, M.T. Mitochondrial respiratory chain supercomplexes are destabilized in Barth syndrome patients. J Mol Biol 361: 462-469, 2006.

Menao, S., López-Viñas, E., Mir, C., Puisac, B., Gratacós, E., Arnedo, M., Carrasco, P., Moreno, S., Ramos, M., Gil, M.C., Pie, A., Ribes, A., Pérez-Cerda, C., Ugarte, M., Clayton, P.T., Korman, S.H., Serra, D., Asins, G., Ramos, F.J., Gómez-Puertas, P., Hegardt, F.G., Casals, N., Pié, J. Ten novel HMGCL mutations in 24 patients of different origin with 3-hydroxy-3-methyl-glutaric aciduria. Hum Mutat 30(3): E520-E529, 2009.

Méndez-Álvarez, E., Soto-Otero, R., Hermida-Aeijeiras, A., López-Real, A.M., Labandeira-García, J.L. Effects of aluminium and zinc on the oxidative stress

caused by 6-hydroxydopamine autoxidation: relevance for the pathogenesis of Parkinson's disease. Biochim Biophys Acta 1586: 155-168, 2001.

Mori N., Yasutake A., Hirayama K. Comparative study of activities in reactive oxygen species production/defense system in mitochondria of rat brain and liver, and their susceptibility to methylmercury toxicity. Arch Toxicol 81: 769-776, 2007.

Nourooz-Zadeh, J., Liu, E.H., Yhlen, B., Anggard, E.E., Halliwell, B. F4-isoprostanes as specific marker of docosahexaenoic acid peroxidation in Alzheimer's disease. J Neurochem 72: 734-740, 1999.

Paxinos, G. & Watson, C. The rat brain in Stereotaxic Coordinates. 2º ed. Sydney: Academic Press, 1986.

Perez-Severiano, F., Rios, C., Segovia, J. Striatal oxidative damage parallels the expression of a neurological phenotype in mice transgenic for the mutation of Huntington's disease. Brain Res 862: 234-237, 2000.

Pié, J., López-Viñas, E., Puisac, B., Menao, S., Pié, A., Casale, C., Ramos, F.J., Hegardt, F.G., Gómez-Puertas, P., Casals, N. Molecular genetics of HMG-CoA lyase deficiency. Mol Genet Metab 92(3): 198-29, 2007.

Pospisilova, E., Mrazova, L., Hrda, J., Martincova, O., Zeman, J. Biochemical and molecular analyses in three patients with 3-hydroxy-3-methylglutaric aciduria. J Inherit Metab Dis 26: 433-441, 2003.

Pushpendran C.K., Subramanian M., Devasagayam T.P. Developmental changes in the peroxidation potential of rat brain homogenate and mitochondria. Mech Ageing Dev 73: 197-208, 1994.

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

Reiter, R.J., Carneiro, R.C., Oh, C.S. 1997a. Melatonin in relation to cellular antioxidative defense mechanisms. Horm Metab Res 29: 363-372, 1997a.

Reiter, R., Tang, L., Garcia, J.J., Munoz-Hoyos, A. Pharmacological actions of melatonin in oxygen radical pathophysiology. Life Sci 60: 2255-2271, 1997b

Reiter, R.J., Tan, D.X., Manchester, L.C., Qi, W. 2001. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. Cell Biochem Biophys 34: 237-256.

Ribeiro, C.A.J., Balestro, F., Grando, V., Wajner, M. Isovaleric acid reduces Na+,K+-ATPase activity in synaptic membranes from cerebral cortex of young rats. Cell and Mol Neurobiol 27 (4), 529-540, 2007.

Rose, C.D., Henneberry, R.C. Etiology of the neurodegenerative disorders: a critical analysis. Neurobiol Aging 15:233-234, 1994.

Ruesch, S., Krähenbühl, S., Kleinle, S., Liechti-Gallati, S., Schaffner, T., Wermuth, B., Weber, J., Wiesmann, U.N. Combined 3-methylglutaconic adn 3-hydroxy-3-methylglutaric aciduria with endocardial fibroelastosis and dilatative

cardiomyopathy in male ad female siblings with partial deficiency of complex II/III in fibroblasts. Enzyme Protein 49(5-6): 321-329, 1996.

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

Stellmer, F., Keyser, B., Burckhardt, B.C., Koepsell, H., Streichert, T., Glatzel, M., Jabs, S., Thiem, J., Herdering, W., Koeller, D.M., Goodman, S.I., Lukacs, Z., Ullrich, K., Burckhardt, G., Braulke, T., Muhlhausen, C. 3-Hydroxyglutaric acid is transported via the sodium-dependent dicarboxylatetransporter NaDC3. J Mol Med 85 (7): 763-770, 2007.

Schapira, A.H., Cooper, J.M., Dexter, D., Jenner, P., Clark, J.B., Marsden, C.D. Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1: 1269, 1989.

Schapira, A.H., Cooper, J.M., Dexter, D., Clark, J.B., Jenner, P., Marsden, C.D. Mitochondrial complex I deficiency in Parkinson’s disease. J Neurochem 54: 823-827, 1990a.

Schapira, A.H., Mann, V.M., Cooper, J.M., Dexter, D., Daniel, S.E., Jenner, P., Clark, J.B., Marsden, C.D. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson’s disease, J Neurochem 55: 2142-2145, 1990b.

Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (editores). The Metabolic and Molecular Bases of Inherited Disease. 8ª edition. New York, McGraw-Hill. 3-45, 2001.

Shoji, Y, Takahashi, T., Sawaishi, Y., Ishida, A., Matsumori, M., Shoji, Y.A., Enoki, M., Watanabe, H., Takada, G. 3-Methylglutaconic aciduria type I: Clinical heterogeneity as a neurometabolic disease. J Inherit Metab Dis 22: 1-8, 1999.

Singh, P., Jain, A., Kaur, G. Impact of hypoglycemia and diabetes on CNS: Correlation of mitochondrial oxidative stress with DNA damage. Mol Cell Biochem 260: 153-159, 2004.

Sitta, A., Barschak, A.G., Deon, M., Terroso, T., Pires, R., Giugliani, R., Dutra-Filho, C.S., Wajner, M., Vargas, C.R. Investigation of oxidative stress parameters in treated phenylketonuric patients.Metab Brain Dis 21: 287-296, 2006.

Smith, C.D., Carney, J.M., Starke-Reed, P.E., Oliver, C.N., Stadtman, E.R., Floyd, R.A., Markesbery, W.R. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. Proc Natl Acad Sci USA 88: 10540-10543, 1991.

Southorn, P.A., Powis, G. Free radicals in medicine. I. Chemical nature and biologic reactions. Mayo Clin Proc 63: 381-389, 1988.

Sperl, W., Jesina, P., Zeman, J., Mayr, J.A., Demeirleir, L., VanCoster, R., pícková, A., Hansiková, H., Houst'ková, H., Krejcík, Z., Koch, J., Smet, J., Muss, W., Holme, E., Houstek, J. Deficiency of mitochondrial ATP synthase of nuclear genetic origin. Neuromuscul Disord 12: 821-829, 2006.

Stoy, N., Mackay, G.M., Forrest, C.M., Christofides, J., Egerton, M., Stone, T.W., Darlington, L.G. Tryptophan metabolism and oxidative stress in patients with Huntington's disease. J Neurochem 93: 611-623, 2005.

Suh, S.W., Gum, E.T., Hamby, A.M., Chan, P.H., Swanson, R.A. Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. J Clin Invest 117(4): 910-918, 2007.

Sweetman, L., Williams, J.C. Branched chain organic acidurias. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D., editores. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill. p. 2340–2342, 2001.

Tranebjaerg, L., Schwartz, C., Eriksen, H., Andreasson, S., Ponjavic, V., Dahl, A., Stevenson, R.E., May, M., Arena, F., Barker, D. A new X linked recessive deafness syndrome with blindness, dystonia, fractures, and mental deficiency is linked to Xq22. J Med Genet 32: 257-263, 1995.

Vargas, C.R., Sitta, A., Schmitt, G., Ferreira, G.C., Cardoso, M.L., Coelho, D., Gibson, K.M., Wajner, M. Incidence of 3-hydroxy-3-methylglutaryl-CoA coenzyme A lyase (HL) deficiency in Brazil, South America. J Inherit Metab Dis, 2007.

Yagi, K. Simple procedure for especific assay of lipid hydroperoxides in serum or plasma. Methods Mol Biol 108: 107-110, 1998.

Yalcinkaya C, Dincer A, Gu¨ndu¨z E, Ficicioglu C, Kocer N, Aydin A. MRI and MRS in HMG-CoA lyase deficiency. Pediatr Neurol 20:375–380, 1999.

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

Watson, M.S., Mann, M.Y., Lloyd-Puyear, M.A., Rinaldo, M. Newborn screening: toward a uniform screening panel and system. Genet Med 8 (1): 1S-252S, 2006.

Wortmann, S., Rodenburg, R.J., Huizing, M., Loupatty, F.J., de Koning, T., Kluijtmans, L.A., Engelke, U., Wevers, R., Smeitink, J.A., Morava, E. Association of 3-methylglutaconic aciduria with sensori-neural deafness, encephalopathy, and Leigh-like syndrome (MEGDEL association) in four patients with a disorder of the oxidative phosphorylation. Mol Genet Metab 88: 47-52, 2006.

Wortmann, S.B., Rodenburg, R.J., Jonckheere, A., de Vries, M.C., Huizing, M., Heldt, K., van den Heuvel, L.P., Wendel, U., Kluijtmans, L.A., Engelke, U.F., Wevers, R.A., Smeitink, J.A., Morava, E. Biochemical and genetic analysis of 3-methylglutaconic aciduria type IV: a diagnostic strategy. Brain 132: 136-146, 2009.

Zaifeiriou, D.I., Vargiami, E., Mayapetek, E., Augoustidou-Savvopoulou, P., Mitchell, G.A. 3-Hydroxy-3-methylglutaryl Coenzyme A lyase deficiency with reversible white matter changes after treatment. Pediatr Neurol 37(1): 47-50, 2007.

Zugno, A.I., Stefanello, F.M., Scherer, E.B., Mattos, C., Pederzolli, C.D., Andrade, V.M., Wannmacher, C.M., Wajner, M., Dutra-Filho, C.S., Wyse, A.T.

Guanidinoacetate decreases antioxidant defenses and total protein sulfhydryl content in striatum of rats. Neurochem Res 33: 1804-1810, 2008.