UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS – GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

ESTUDOS SOBRE A HOMEOSTASE REDOX EM UM MODELO ANIMAL DE ACIDEMIA GLUTÁRICA TIPO I

Bianca Seminotti

ORIENTADOR: Prof. Dr. Moacir Wajner

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas - Bioquímica como requisito parcial à obtenção do grau de Doutor em Bioquímica.

Porto Alegre, 2014

“Mas o homem, porque não tem senão uma vida,

não tem nenhuma possibilidade de verificar a hipótese através de

experimentos, de maneira que não saberá nunca se errou ou acertou

ao obedecer a um sentimento.”

(Milan Kundera, “A insustentável leveza do ser”)

CIP - Catalogação na Publicação

Seminotti, Bianca
Estudos sobre a homeostase redox em um modelo animal para academia glutárica tipo I / Bianca Seminotti. -- 2014.
134 f.

Orientador: Moacir Wajner.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2014.

1. Estresse Oxidativo. 2. Erros Inatos do Metabolismo. 3. Acidemia Glutárica tipo I. I. Wajner, Moacir, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

À Deus,

Aquele que é poderoso para fazer

infinitamente mais do que tudo quanto pedimos ou pensamos.

(Efésios 3.20)

AGRADECIMENTOS

A Universidade Federal do Rio Grande do Sul, pelo ensino de qualidade,
e por proporcionar experiências únicas.

Ao Departamento de Bioquímica, por conceder as condições para a
realização deste trabalho, e aos professores e funcionários,
por todo o suporte necessário.

Aos professores do grupo de Erros Inatos do Metabolismo,
pela convivência agradável e pela ajuda sempre que necessária.

Ao meu orientador professor Moacir, que me acolheu há 8 anos atrás,
por compartilhar experiências e conhecimento, e pelo carinho.

Aos colegas do laboratório 38, César, Alexandre, Carol Viegas, Valeska,
Estela, Ane, Carol Fernandes, Ânge, Mateus Struecker, Duda e Lu,
pelo auxílio e pela convivência agradável.

Aos bolsistas de iniciação científica, Cris, Clarissa, Rafa Martell, Wagner,
Dani, Carol Coffi, por ajudarem sempre, e em especial ao Rafa,
que fez deste trabalho o seu também.

Aos colegas do laboratório 27, Alana, Mateus, Bel, Anna, Léo e Gustavo,
pela disposição em ajudar e pelo companheirismo.

Aos que já passaram pelo laboratório,
Rafa, Lali, Alexandre Solano, Anna Laura, Ana Paula e Doda,
que me ensinaram muito e deixaram saudades.

Aos queridos amigos da faculdade, Dudu, Lari, Linda e Biel,
por se importarem e pelos momentos compartilhados.

Às minhas "Lu's", Luciana e Lucila, 001 e 002,
por me acolherem na humilde residência, e também em seus corações.

À Martha, amiga única e especial, por continuar sendo um porto seguro,
agora um pouco longe, e mesmo assim sempre perto.

Mais uma vez, aos meus antigos e preciosos amigos, por tudo que vivemos
durante o tempo em que estivemos mais perto. Sinto saudades.

Ao Gui, meu namorado e meu melhor amigo, pelo cuidado e pelo carinho,
e por compartilhar sua vida comigo.

À minha família, minha âncora, pelo incentivo e pelo apoio.

Ao Pablo, meu irmão querido e meu gurizinho, pela paciência,
pelos mimos, e por tornar minha vida mais leve.

Aos meus pais, minha saxofonista e meu gaiteiro,
meus exemplos de vida, pelo amor, educação, dedicação, confiança
e pelo amparo em cada momento da minha vida.

À Deus, pela minha vida, por ser meu refúgio e pelo colo de Pai,
e pela bênção de ter pessoas maravilhosas em minha vida.

SUMÁRIO

PARTE I

Introdução e Objetivos

RESUMO

A acidemia glutárica tipo I (AG I) causada pela deficiência severa da enzima glutaril-CoA desidrogenase, que catalisa a degradação de lisina (Lis), resulta no acúmulo predominante dos ácidos glutárico (AG) e 3-hidroxiglutárico (3HG). Visto que os mecanismos responsáveis pelos danos cortical e estriatal observados nos pacientes ainda não estão totalmente esclarecidos, avaliamos a homeostase redox em diferentes regiões cerebrais (córtex cerebral, estriado e hipocampo) e tecidos periféricos (fígado e coração) de camundongos selvagens (*Gcdh*$^{+/+}$) e nocaute para a enzima glutaril-CoA desidrogenase (*Gcdh*$^{-/-}$, modelo genético da AG I) com 15, 30 e 60 dias de vida. Os animais foram submetidos a uma sobrecarga de Lis através de uma injeção intraperitoneal (8 µmol/g) ou de uma dieta enriquecida com esse aminoácido (2,8 % ou 4,7% de Lis) por 60 horas ou 40 dias (2,8% de Lis). Determinamos as substâncias reativas ao ácido tiobarbitúrico (TBA-RS), conteúdo de grupamentos sulfidrilas e formação de grupamentos carbonila, nitratos e nitritos e oxidação da 2',7'- diclorofluoresceína (DCFH), concentrações de glutationa reduzida (GSH), bem como as atividades das enzimas antioxidantes glutationa peroxidase, glutationa redutase, superóxido dismutase, catalase e glicose-6-fosfato desidrogenase. Inicialmente verificamos que os grupamentos sulfidrilas estavam reduzidas em cérebro e as concentrações de GSH estavam diminuidas em fígado de animais *Gcdh*$^{-/-}$ de 15 dias, quando comparados aos animais *Gcdh*$^{+/+}$ alimentados com uma dieta padrão. A injeção aguda de Lis, além de diminuir os níveis de GSH e os grupamentos tióis em animais *Gcdh*$^{-/-}$, aumentou a formação de carbonilas e a oxidação de DCFH, bem como alterou as atividades das enzimas antioxidantes em cérebro e fígado, quando comparados aos animais *Gcdh*$^{+/+}$ que também receberam Lis. Por outro lado, os parâmetros avaliados em camundongos de 30 dias de vida não diferiram no cérebro e tecidos periféricos dos animais *Gcdh*$^{-/-}$ em relação aos camundongos selvagens alimentados com uma dieta padrão. A injeção aguda de Lis em animais *Gcdh*$^{-/-}$ de 30 dias de vida provocou um aumento das concentrações cerebrais de AG e 3HG, sendo aproximadamente 40% mais altas no estriado quando comparadas com as observadas em córtex cerebral. A injeção aguda de Lis e a administração da dieta enriquecida com Lis em animais *Gcdh*$^{-/-}$ de 30 dias provocaram dano oxidativo lipídico e proteico e alteraram as defesas antioxidantes em estriado e córtex cerebral, mas não nos outros tecidos desses camundongos com relação aos animais *Gcdh*$^{+/+}$. Além disso, animais *Gcdh*$^{-/-}$ de 60 dias de vida tratados com dieta padrão ou dieta enriquecida com Lis (2,8%) por 40 dias, a partir do 21º dia de vida, mostraram apenas um aumento nos níveis de TBA-RS em córtex cerebral e estriado, enquanto que outros parâmetros avaliados não foram significativamente alterados. Finalmente, através de coloração com hematoxilina e eosina, observou-se a presença de vacúolos no estriado de animais *Gcdh*$^{-/-}$ de 60 dias de vida após 40 dias de dieta rica em Lis (2,8%). Já em córtex cerebral se encontraram vacúolos nos animais *Gcdh*$^{-/-}$ com dieta padrão, o que não foi acentuado pela sobrecarga de Lis. Concluindo, presumimos que uma alteração da homeostase redox celular causada por sobrecarga de Lis, principalmente nos estágios iniciais do desenvolvimento, possa contribuir para a patogênese do dano cerebral particularmente estriatal na AG I.

ABSTRACT

Glutaric acidemia type I (GA I) is an organic acidemia caused by the severe deficiency of glutaryl-CoA dehydrogenase, that catalyzes the degradation of lysine (Lys), biochemically characterized by the predominant accumulation of glutaric (GA) and 3-hydroxyglutaric (3HG) acids in tissues and biological fluids of patients. Considering that the mechanisms underlying the cortical and striatal damage observed in affected individuals remain unclear, we assessed parameters of redox homeostasis in different brain regions (cerebral cortex, striatum and hippocampus) and peripheral tissues (liver and heart) of wild-type (*Gcdh*$^{+/+}$) and knockout mice for the enzyme glutaryl-CoA dehydrogenase (*Gcdh*$^{-/-}$, a genetic model of GA I) with 15, 30 and 60 days old. The animals were submitted to an overload of Lys through a single intraperitoneal injection (8 umol / g) or to an enriched diet with this amino acid (2.8% or 4.7 % Lys) for 60 hours or 40 days (2.8% Lys). We determined the levels of thiobarbituric acid reactive substances (TBA-RS), concentrations of sulfhydryl and carbonyl groups, nitrates and nitrites and oxidation of 2',7'-dichlorofluorescein (DCFH), concentrations of reduced glutathione (GSH) as well as the activities of the antioxidant enzymes glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase and glucose-6-phosphate dehydrogenase. We initially found that the sulfhydryl groups were reduced in the brain and GSH concentrations were decreased in the liver of 15-day-old *Gcdh*$^{-/-}$ animals, compared to *Gcdh*$^{+/+}$ animals. Besides decreasing the concentrations of GSH and thiol groups in *Gcdh*$^{-/-}$ mice, Lys acute injection also increased carbonyl formation and DCFH oxidation, and altered the profile of antioxidant enzyme activities in brain and liver, when compared to *Gcdh*$^{+/+}$ mice receiving Lys. The parameters measured in 30-day-old *Gcdh*$^{-/-}$ mice did not differ in brain and peripheral tissues, as compared to wild type mice treated with normal diet. However, an acute injection of Lys caused an increase of GA and 3HG concentrations in the brain, approximately 40 % higher in the striatum, when compared to the cerebral cortex levels. The Lys acute injection and the enriched Lys diet caused protein oxidative damage and altered both enzymatic and non-enzymatic antioxidant defenses in striatum and cerebral cortex of *Gcdh*$^{-/-}$ mice, but not in other tissues of these mice, when compared to the wild type mice. Furthermore, 60-day-old *Gcdh*$^{-/-}$ mice treated with a standard or a Lys enriched diet (2.8 %) for 40 days (from the 21th day of life) showed only an increase of TBA-RS levels in cerebral cortex and striatum, whereas the other parameters analyzed were not altered. Finally, histological brain sections of 60-day-old mice, stained with hematoxilin and eosin, showed the presence of vacuoles in striatum of *Gcdh*$^{-/-}$ animals 40 days after the diet supplemented with Lys (2.8%). In the cerebral cortex, vacuoles were observed in *Gcdh*$^{-/-}$ animals with standard diet, but this was not accentuated by Lys overload. In conclusion, it may be presumed that alterations in the cellular redox homeostasis caused by Lys overload, especially in the early stages of development, contribute to the pathogenesis of brain damage observed in GA I, particularly in striatum.

LISTA DE ABREVIATURAS

AG I – acidemia glutárica tipo I;

AG – ácido glutárico;

BHE – barreira hematoencefálica;

CAT – catalase;

CK – creatina quinase;

EIM – erros inatos do metabolismo;

ERN – espécies reativas de nitrogênio;

ERO – espécies reativas de oxigênio;

GABA – ácido gama-aminobutírico;

GCDH – glutaril-CoA desidrogenase;

Gcdh-/- – camundongos nocautes para glutaril-CoA desidrogenase;

GSH – glutationa reduzida;

G6PDH – glicose-6-fosfato desidrogenase;

GPx – glutationa peroxidase;

GR – glutationa redutase;

HE – hematoxilina-eosina;

3HG – ácido 3-hidróxiglutárico;

H_2O – água;

H_2O_2 – peróxido de hidrogênio;

LDL – lipoproteína de baixa densidade;

Lis – lisina;

NADPH – nicotinamida adenina dinucleotídeo fosfato reduzido;

$NO^{\cdot}$ – óxido nítrico;

O_2 – oxigênio molecular;

$O_2^{\cdot-}$ – ânion superóxido;

$OH^{\cdot}$ – radical hidroxila;

$^1O_2$ – oxigênio singlete;

$ONOO^-$ – peroxinitrito;

SOD – superóxido dismutase;

TBA-RS – substâncias reativas ao ácido tiobarbitúrico;

SNC – sistema nervoso central;

I.1. INTRODUÇÃO

I.1.1. Erros Inatos do Metabolismo

Sir Archibald E. Garrod usou o termo erros inatos do metabolismo (EIM) em 1908 para designar doenças como a alcaptonúria em que os indivíduos afetados excretam grandes quantidades de ácido homogentísico na urina. Garrod observou uma maior frequência desta doença em indivíduos de uma mesma família e maior incidência de consanguinidade 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 proteica, 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). Presume-se que em consequência deste bloqueio metabólico possa 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.

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 em seu conjunto afetam aproximadamente 1 a cada 500-2.000 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 frequência destas doenças na população em geral é pouco conhecida, o que pode ser creditado à falta de laboratórios especializados para o seu diagnóstico e ao desconhecimento médico sobre essas enfermidades. Na Holanda, país considerado referência para o diagnóstico de EIM, a incidência 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 consanguinidade é elevada, a frequência é de 1: 740 nascidos vivos (Rashed *et al.*, 1994). Chalmers e colaboradores (1980) demonstraram que as acidemias orgânicas eram os EIM mais frequentes em crianças hospitalizadas motivando diversos estudos clínicos, laboratoriais e epidemiológicos nos anos seguintes.

Os pacientes afetados por acidemias orgânicas apresentam predominantemente disfunção neurológica em suas mais diversas formas de manifestação, que incluem regressão neurológica, convulsões, coma, ataxia, hipotonia, hipertonia, irritabilidade, tremores, movimentos coreatetóticos,

tetraparesia espástica, atraso no desenvolvimento psicomotor, retardo mental, dentre outras. As alterações laboratoriais mais comuns são cetose, cetonúria, neutropenia, trombocitopenia, acidose metabólica, baixos níveis de bicarbonato, hiperglicinemia, hiperamonemia, hipo / hiperglicemia, acidose lática e aumento dos níveis séricos de ácidos graxos livres (Scriver *et al.*, 2001). Com o uso da tomografia computadorizada, na maioria dos pacientes afetados por essas doenças são encontradas alterações de substância branca (hipomielização e / ou desmielinização), atrofia cerebral generalizada ou dos gânglios da base (necrose ou calcificação), macrocefalia, atrofia frontotemporal e atrofia cerebelar (Mayatepek *et al.*, 1996).

I.1.3. Acidemia Glutárica Tipo I

A acidemia glutárica tipo I (AG I, OMIM # 231670) é uma doença neurometabólica autossômica recessiva, primeiramente descrita por Goodman e colaboradores (1975), causada pela deficiência severa na atividade da enzima mitocondrial glutaril-CoA desidrogenase (GCDH, EC 1.3.99.7) da via catabólica dos aminoácidos L-lisina (Lis), L-hidroxilisina e L-triptofano. A reação catalisada por esta enzima ocorre em duas etapas consecutivas: a desidrogenação de glutaril-CoA a glutaconil-CoA e a descarboxilação de glutaconil-CoA a crotonil-CoA (Härtel *et al*., 1993). O composto intermediário glutaconil-CoA pode ser clivado e excretado como ácido glutacônico ou hidratado para 3-hidroxiglutaril-CoA (Figura 1.1).

A atividade deficiente da GCDH compromete o catabolismo dos aminoácidos Lis, L-hidroxilisina e L-triptofano, formando rotas metabólicas alternativas com o acúmulo predominante dos ácidos glutárico (AG) e 3-

hidroxiglutárico (3HG), e também glutacônico, nos tecidos e líquidos biológicos (líquor, plasma e urina) dos pacientes (Frerman, 2001).

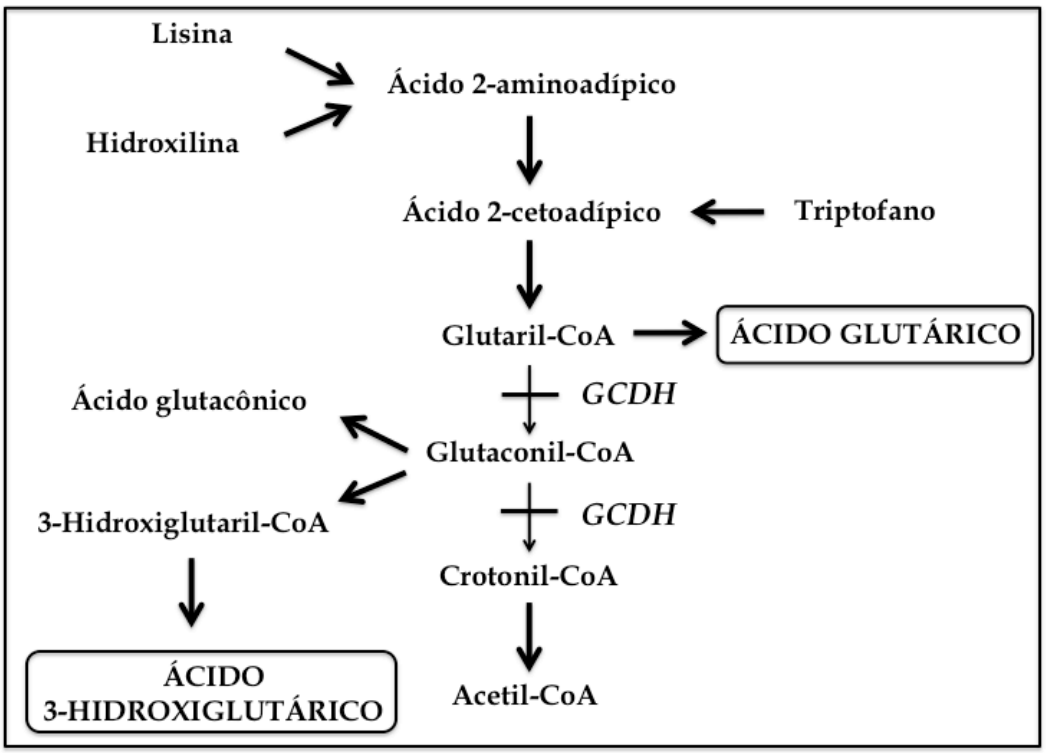


Figura 1.1 – Rota de catabolismo dos aminoácidos triptofano, hidroxilisina e lisina. Adaptado de Scriver *et al*., 2001.

As concentrações no plasma desses ácidos variam entre 5 e 400 µmol/L (Hoffmann *et al*., 1991, 1996; Merinero *et al*., 1995), enquanto que as concentrações cerebrais podem atingir 500–5000 µmol/L para o AG e 40–200 µmol/L para o 3HG (Sauer *et al*., 2006), aumentando ainda mais durante as crises encefalopáticas (Kölker *et al*., 2004). Considerando que a barreira hematoencefélica (BHE) é pouco permeável a esses ácidos orgânicos, acredita-se que a produção desses metabólitos nas células neurais e o seu acúmulo no sistema nervoso central (SNC) possam explicar as diferenças nas concentrações cerebrais e plasmáticas, além de constituir um fator de

risco para a neurodegeneração característica da doença (Hoffmann *et al*., 1993; Kölker *et al*., 2006a, b; Sauer *et al*. 2006; Strauss e Morton, 2003; Strauss *et al*., 2003).

A AG I tem sua prevalência estimada em 1: 30.000-1:100.000 nascidos vivos, sendo considerada umas das mais frequentes doenças do metabolismo dos ácidos orgânicos, podendo atingir uma alta prevalência (até 1: 3.000 nascidos vivos) em algumas comunidades geneticamente homogêneas (Goodman *et al.*, 2001; Kölker *et al.*, 2007).

I.1.3.1. Achados Clínicos e Neuropatológicos

A AG I pertence a um grupo de doenças em que os sintomas são essencialmente neurológicos, sendo considerada por isso uma acidemia orgânica "cerebral". O quadro clínico dos pacientes com AG I é heterogêneo e o curso da doença parece ser seguido por diferentes padrões. Alguns pacientes desenvolvem-se normalmente, enquanto a maioria apresenta hipotonia e atraso progressivo no desenvolvimento, além de crises encefalopáticas. O mais importante achado clínico dos pacientes antes das crises é a macrocefalia progressiva e atrofia cortical fronto-temporal (Hoffmann *et al.*, 1996).

Entre 3 e 36 meses de idade, uma característica degeneração bilateral aguda dos gânglios da base acomete os pacientes durante as crises encefalopáticas, geralmente precipitadas por infecções ou imunizações de rotina (situações em que o paciente se encontra em catabolismo acelerado). As crises encefalopáticas usualmente duram poucos dias, mas cada episódio leva à morte neuronal massiva. Após essas crises agudas, surgem sintomas

relacionados à destruição estriatal, tais como distonia e discinesia, hipotonia, convulsões, rigidez muscular e espasticidade (Goodman *et al.*, 2001; Hoffmann e Zschocke, 1999; Kölker *et al.*, 2008). Isso sugere uma "janela de vulnerabilidade" para o aparecimento dos sintomas, provavelmente relacionada ao período de desenvolvimento cerebral.

Os achados neuropatológicos incluem também atrofia cortical fronto-temporal ao nascimento, formação espongiforme e diminuição de substância branca (hipomielinização ou desmielinização) progressiva e uma característica degeneração bilateral aguda dos gânglios da base durante os episódios de descompensação metabólica entre os 6 e os 36 meses de idade (Brismar e Ozand, 1995; Couce *et al*., 2013; Hoffmann e Zschocke, 1999; Neumaier-Probst *et al*., 2004; Nunes *et al*., 2013; Olivera-Bravo *et al*., 2013; Strauss *et al*., 2003).

Além disso, exames de imagem por ressonância magnética geralmente mostram alterações espongiformes progressivas na substância branca (leucoencefalopatia) com hipoplasia cortical e vacuolização, hemorragia subdural e degeneração dos gânglios da base (Bodamer *et al*., 2004; Carman *et al*., 2012; Couce *et al*., 2013; Goodman *et al*., 1977; Harting *et al*., 2009; Hedlund *et al*., 2006; Neumaier-Probst *et al*., 2004; Nunes *et al*., 2013; Pérez-Dueñas *et al*., 2009; Strauss e Morton, 2003). Também podem ser observados um alargamento dos espaços subaracnóideos da região frontotemporal e uma difusa atenuação da substância branca periventricular (Drigo *et al.*, 1996; Pineda *et al.*, 1998), tornando as crianças afetadas mais propensas a sofrerem hemorragias agudas subdurais após traumas de

pequena intensidade na cabeça (Hoffmann e Zschocke, 1999; Zinnanti *et al*., 2014).

I.1.3.2. Aspectos Moleculares

Localizado no cromossomo 19p 13.2, o gene da GCDH codifica um polipeptídeo de 438 aminoácidos que, após uma clivagem na porção *N*-terminal retirando 44 aminoácidos, forma a proteína madura dentro da matriz mitocondrial (Goodman *et al*., 1998). A maioria das mutações descritas está relacionada com simples mudanças de bases, como no caso da mais frequente mutação em caucasianos R402W que acomete entre 10 e 20% dos alelos (Busquets *et al.*, 2000; Goodman *et al.*, 1998; Zschocke *et al.*, 2000).

A deficiência da GCDH é muito heterogênea, e muitos dos pacientes são heterozigotos para duas diferentes mutações, mas dentro de comunidades específicas o padrão é mais homogêneo (Busquets *et al*., 2000). Apesar do conhecimento de diferentes mutações, não há correlação entre o genótipo e a atividade enzimática, bem como o fenótipo bioquímico, clínico e o prognóstico dos pacientes (Goodman *et al*., 1998; Hoffmann e Zschocke, 1999; Kölker *et al*., 2006a).

I.1.3.3. Diagnóstico

O diagnóstico de AG I é feito através da detecção do aumento da excreção urinária dos ácidos AG) e 3HG, sendo identificados por cromatografia gasosa acoplada à espectrometria de massa (Hoffmann *et al*., 1993, Kölker *et al*., 2012). Em alguns casos, a confirmação do diagnóstico é feita através da medida da atividade enzimática em fibroblastos de pacientes.

Outro método complementar de diagnóstico é a análise do perfil de acilcarnitinas, onde podem ser observadas elevações de glutarilcarnitina, além de uma diminuição dos níveis de carnitina livre, através de espectrometria de massas em tandem MS/MS nos líquidos biológicos dos pacientes (Ziadeh *et al.*, 1995). A análise mutacional pode auxiliar no diagnóstico, porém, devido ao grande número de mutações conhecidas, este método tem um melhor aproveitamento em comunidades fechadas, onde as mutações se repetem em uma maior proporção (Strauss *et al*., 2003).

Em alguns casos, pacientes com AG I podem apresentar excreção urinária pouco elevada ou intermitente, ou até mesmo excreção normal do AG (não excretores) (Bergman *et al.*, 1989; Campistol *et al.*, 1992; Hoffmann, 1996; Merinero *et al.*, 1995). Nesses casos, se houver a suspeita da AG I por achados clínicos e/ou neuroradiológicos, a medida da atividade da enzima GCDH em fibroblastos (ou leucócitos) se torna necessária (Goodman e Frerman, 2001). A importância do diagnóstico neonatal precoce da AG I através dos testes de triagem já realizados em alguns países e do início imediato do tratamento é importante para a prevenção das crises encefalopáticas e suas consequências fatais (Lee *et al*., 2013; Lindner *et al*., 2006; Pfeil *et al*., 2013; Viau *et al*., 2012).

I.1.3.4. Tratamento

O tratamento dos pacientes com AG I baseia-se fundamentalmente na restrição de Lis da dieta e administração de L-carnitina e riboflavina (Gokmen *et al*., 2012). Além disso, o tratamento nas crises agudas, durante as infecções, consiste na administração de líquidos para evitar desidratação e

na ingesta hipercalórica para controlar o catabolismo, além de outras medidas gerais. A administração de insulina, glicose e bicarbonato também pode prevenir ou diminuir o dano estriatal durante estes episódios, por inibir o catabolismo e a acidose severa (Boy *et al*., 2013).

Devido à inibição da glutamato descarboxilase neuronal e às baixas concentrações de GABA nos gânglios da base dos pacientes (Leibel *et al*., 1980), o tratamento com o ácido valpróico foi utilizado em alguns pacientes, pois este composto leva a um aumento seletivo de GABA nas sinapses (através da inibição da GABA transaminase ou da semialdeído succinil desidrogenase) (Stutchfield *et al.,* 1985). No entanto, enfatize-se que, se o dano estriatal se estabelecer após uma ou mais crises encefalopáticas, o tratamento posterior poderá ser insatisfatório.

Outro tratamento proposto é o uso de drogas anticolinérgicas, como triexifenidil e toxina botulínica, para minimizar a discinesia e a distonia encontradas em pacientes após as crises encefalopáticas (Burlina *et al*., 2004). Contudo, os anticolinérgicos e os anticonvulsivantes não mostraram resultados satisfatórios em longo prazo (Hoffmann *et al*., 1996; Hoffmann e Zschocke, 1999; Yamaguchi *et al*., 1987). Dessa forma, alternativas têm sido propostas e avaliadas. Baseados em estudos prévios em um modelo animal de AG I (Zinnanti *el al*., 2006), Zinnanti e colaboradores (2007) recentemente propuseram a administração de glicose e homoarginina aos pacientes. O objetivo foi o de reduzir o acúmulo cerebral dos metabólitos tóxicos produzidos pela deficiência da GCDH, visto que a arginina irá diminuir a entrada da Lis através da BHE por competição pelo sistema de transporte Y (Kölker *et al*., 2012; Sauer *et al*., 2011). Desde então a suplementação

dietética com arginina vem sendo usada para tratar indivíduos acometidos pela AG I, e estudos recentes têm relatado o benefício dessa dieta (Kölker *et al*., 2012; Sauer *et al*., 2011; Strauss *et al*., 2011).

I.1.3.5. Fisiopatologia

Nos últimos anos, distintos mecanismos têm sido propostos para explicar a fisiopatogenia do dano cerebral da AG I. Apesar de algumas investigações prévias terem sugerido efeitos tóxicos do AG e do 3HG, até o presente momento, os mecanismos fisiopatológicos que levam ao dano cortical e estriatal apresentado pelos pacientes afetados pela AG I ainda não foram totalmente elucidados.

Diversos autores relacionam as vacuolizações encontradas em estudos *postmortem* de cérebro de pacientes afetados pela deficiência da GCDH à excitotoxicidade (Goodman *et al.*, 1977; Hoffmann e Zschocke, 1999) e consequente dano celular (Danbolt, 2001). Esta hipótese está baseada na similaridade estrutural existente entre o glutamato e os principais ácidos orgânicos (AG e 3HG) (Flott-Rahmel *et al.*, 1997; Wajner *et al.*, 2004). Nesse sentido, vários trabalhos tentam explicar a neuropatologia da AG I pela interação desses ácidos orgânicos com receptores e transportadores glutamatérgicos (Bjugstad *et al.*, 2001; Dalcin *et al*., 2007; Kölker *et al.*, 2008; Porciúncula *et al.*, 2000; Porciúncula *et al.*, 2004; Rosa *et al.*, 2004; Wajner *et al*., 2004). No entanto, apesar de diversas evidências da neurotoxicidade destas substâncias relacionadas ao sistema glutamatérgico, outros trabalhos não confirmam essa hipótese (Freudenberg *et al*., 2004; Lund *et al.*, 2004), fazendo com que esta questão continue em intenso debate.

Alterações da bioenergética também têm sido propostas para explicar o dano cerebral na AG I. Estudos *in* vitro demonstraram alteração da homeostase energética pelos ácidos AG e 3HG em cérebro e culturas de neurônios de ratos (da Costa Ferreira *et al*., 2005; Ferreira *et al*., 2007; Kölker *et al*., 2002a, 2002b; Latini *et al*., 2005a, Silva *et al*., 2000; Ulrich *et al*., 1999). Além disso, estudos *in vivo* de ratos tratados cronicamente com AG mostraram inibições nas atividades dos complexos I-III, II, II-III e da enzima creatina quinase (CK) (Ferreira *et al*., 2005, 2007). Uma inibição da enzima Na^+, K^+ - ATPase pelo AG foi observada em córtex cerebral de ratos *in vitro* (Kölker *et al*., 2002b), assim como em cérebro de ratos tratados cronicamente ou através de uma injeção intraestriatal desse ácido orgânico (Fighera *et al*., 2006; Rodrigues *et al*., 2013), e a presença de disfunção mitocondrial foi demonstrada em cultura de astrócitos de ratos tratados com AG ou 3HG (Olivera *et al*., 2008).

Além disso, alterações do estado redox causadas pelo AG e 3HG foram observadas através de estudos *in vivo* e *in vitro*. Neste contexto, foi verificado que tanto o AG (de Oliveira Marques *et al*., 2003; Magni *et al*., 2009) como o 3HG (Latini *et al*., 2002, 2005b) aumentam a lipoperoxidação e diminuem as defesas antioxidantes e os níveis de glutationa reduzida (GSH) em cérebro de ratos *in vitro*. A geração de espécies reativas de oxigênio (ERO) na presença de 3HG também foi evidenciada em culturas de neurônios de telencéfalos de embriões de pinto (Kolker *et al*., 2001) e em astrócitos de linhagem celular C6 (Quincozes-Santos *et al*., 2010). Também foi demonstrado que a administração aguda e crônica de AG aumenta a peroxidação lipídica e diminui as defesas antioxidantes em diferentes

estruturas cerebrais, fígado e eritrócitos de ratos (Fighera *et al*., 2006; Latini *et al*., 2007; Magni *et al*., 2012; Rodrigues *et al*., 2013).

Outros trabalhos demonstraram uma disfunção endotelial com perda da integridade da BHE causada pelos metabólitos acumulados na AG I (Muhlhausen *et al*., 2006: Strauss e Morton, 2003: Zinnanti *et al*., 2006; Zinnanti *et al*., 2014), sendo também proposto que os metabólitos da via das quinureninas, uma das rotas de catabolismo do triptofano, estejam associados com o dano cerebral nessa doença (Heyes 1987: Lehnert e Sass, 2005: Varadkar e Surtees, 2004).

Enfatize-se que os resultados mencionados acima são oriundos de estudos *in vitro* e *in vivo* em ratos selvagens ou em culturas de células obtidas de animais com atividade normal da GCDH.

Recentemente, um novo modelo de AG I foi concebido, onde a enzima GCDH está inativa através da alteração do gene responsável por codificá-la (modelo nocaute) (Koeller *et al*., 2002). Esse modelo animal de AG I tem sido considerado o melhor modelo disponível dessa patologia. Estudos recentes com esse modelo demonstraram que o transporte de sucinato dos astrócitos para os neurônios foi inibido pela ação do AG e 3HG sobre o transportador de dicarboxilatos em culturas primárias de astrócitos e neurônios de camundongos *Gcdh*$^{-/-}$ (Lamp *et al*., 2011). Zinnanti e colaboradores encontraram uma diminuição nos níveis de ATP, fosfocreatina, α-cetoglutarato, CoA, glutamato e GABA, bem como um aumento de acetil-CoA em cérebro de camundongos *Gcdh*$^{-/-}$ (Zinnanti *et al*., 2007).

Trabalhos recentes publicados com o modelo nocaute para a enzima GCDH também mostraram que camundongos *Gcdh*$^{-/-}$ adultos apresentaram

alterações comportamentais com perda da memória de curta e longa duração (Busanello *et al.*, 2012), bem como redução na atividade e na expressão da enzima Na^+,K^+ - ATPase e na atividade da enzima CK em córtex cerebral (Amaral *et al.*, 2012a). Animais *Gcdh*$^{-/-}$ mais jovens (15 dias de vida) expostos a uma sobrecarga de Lis apresentaram um distúrbio na bioenergética celular, observado através da inibição da atividade das enzimas C e Na^+,K^+ - ATPase (Amaral *et al.*, 2012b). Além disso, se observou acidente vascular cerebral metabólico e hemorragia cerebral espontânea após uma sobrecarga de Lis nesse mesmo modelo para a AG I, indicando uma ruptura da integridade da BHE, bem como perda de sua funcionalidade (Zinnanti *et al*., 2014).

I.1.3.6. Modelos Animais de Acidemia Glutárica Tipo I

O desenvolvimento de modelos animais que reproduzam as alterações bioquímico-metabólicas e os achados neuropatológicos da AG I têm sido o foco de diversos estudos, constituindo-se em um desafio para a elucidação da patogênese desse distúrbio. Ferreira e colaboradores (2005) propuseram um modelo químico em ratos, administrando por via subcutânea AG diariamente do 7^o ao 22^o dia de vida, em que altas concentrações desse ácido orgânico foram encontradas no cérebro.

Além disso, um modelo de degeneração estriatal aguda proposto for Strauss e Morton utiliza o ácido 3-nitropropiônico (Strauss e Morton, 2003), um inibidor clássico do complexo II da cadeia respiratória empregado em modelos de doença de Huntington, que apresenta características neurorradiológicas idênticas às observadas em pacientes com AG I.

Com relação ao modelo nocaute estabelecido em camundongos por Koeller *et al.* (2002), foi verificado que os animais apresentaram um fenótipo bioquímico similar ao dos pacientes, com elevados níveis de AG, 3HG e conjugados de glicina e carnitina. Contudo, esse modelo não reproduziu o fenótipo neurológico e a degeneração estriatal característica dos pacientes afetados. Um aperfeiçoamento deste modelo foi proposto por Zinnanti e colaboradores (2006) com a administração, via oral, de uma sobrecarga de Lis aos animais. Foi verificado nesse caso que as concentrações de AG no cérebro dos camundongos *Gcdh*$^{-/-}$ aumentaram significativamente e que os mesmos apresentaram um padrão de neurodegeneração dependente do estágio de desenvolvimento, semelhante ao apresentado pelos pacientes (lesão estriatal aguda e leucoencaflopatia progressiva), além de provocar a perda de seletividade da BHE. Os mesmos autores demonstraram que essa sobrecarga de Lis provoca disfunção mitocondrial e propuseram a utilização da suplementação com glicose e homoarginina para reduzir o acúmulo cerebral dos metabólitos tóxicos gerados pela deficiência da GCDH (Zinnanti *et al.*, 2007). Apesar da intensa investigação e do grande número de trabalhos buscando compreender a patogênese da AG I, as causas da suscetibilidade do córtex fronto-temporal durante a gestação e da janela de vulnerabilidade estriatal durante os primeiros anos de vida permanecem obscuras, constituindo-se nos principais desafios da pesquisa da AG I (Goodman, 2004).

I.1.4. Radicais Livres

Radicais livres são espécies químicas com um ou mais elétrons desemparelhados e possuem a capacidade de existir de forma independente (Halliwell e Gutteridge, 2007; Southorn e Powis, 1988). Essas espécies químicas podem ser átomos, metais de transição ou uma molécula onde o elétron desemparelhado esteja no orbital externo. Este elétron confere uma alta reatividade à molécula, pois pode perder ou adquirir um segundo elétron para esse orbital (Halliwell, 2006).

Os radicais livres podem ser formados pela perda de um elétron de um não-radical ou pelo ganho de um elétron por um não-radical. Radicais podem também ser formados em um processo de fissão homolítica, no qual uma ligação covalente é quebrada e cada elétron do par compartilhado permanece com cada um dos átomos envolvidos (Halliwell e Gutteridge, 2007). Quando um radical livre reage com um composto não-radical, outro radical livre pode ser formado; assim, a presença de um único radical pode iniciar uma sequência de reações em cadeia de transferência de elétrons (redox) (Maxwell, 1995). Nas reações em cadeia induzidas pelos radicais livres, um radical reativo leva à formação de um produto que também é um radical livre e que, por sua vez, reage produzindo um terceiro radical.

Em condições fisiológicas do metabolismo celular aeróbio, o oxigênio molecular (O_2) sofre redução tetravalente, por receber quatro elétrons, resultando na formação de água (H_2O). No entanto, aproximadamente 2% do oxigênio utilizado na cadeia de transporte de elétrons mitocondrial não é completamente reduzido à água, podendo ser convertido a intermediários reativos, como os radicais superóxido ($O_2^{\cdot-}$) e hidroxila ($OH^{\cdot}$), e também o

peróxido de hidrogênio (H_2O_2), processo esse que pode ser exacerbado em condições patológicas (Boveris e Chance, 1973; Roede e Jones, 2010).

O termo genérico espécies reativas de oxigênio (ERO) é usado para incluir não só os radicais formados pela redução do O_2 ($O_2^{\cdot-}$ e $OH^{\cdot}$), mas também alguns não-radicais derivados do oxigênio, como o peróxido de hidrogênio (H_2O_2) e o oxigênio *singlet* ($^1O_2$) (Halliwell e Guteridge, 2007). Além dessas, existem ainda as espécies reativas de nitrogênio (ERN), sendo o óxido nítrico ($NO^{\cdot}$) e o peroxinitrito ($ONOO^-$) as principais representantes.

I.1.5. Defesas Antioxidantes

Antioxidantes são substâncias endógenas ou exógenas que reduzem a formação de radicais livres ou reagem com os mesmos, neutralizando-os. Embora diferindo na composição, as defesas antioxidantes estão amplamente distribuídas no organismo e compreendem agentes que removem cataliticamente os radicais livres, como as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR) e glicose-6-fosfato desidrogenase (G6PDH), dentre outras; proteínas que minimizam a disponibilidade de pró-oxidantes (íons de ferro e cobre, por exemplo) ao se ligarem aos mesmos, como as transferrinas; proteínas que protegem biomoléculas de danos (incluindo dano oxidativo) por outros mecanismos; agentes de baixo peso molecular que aprisionam ERO e ERN, como glutationa reduzida (GSH), α-tocoferol, ácido ascórbico e a bilirrubina (Halliwell e Gutteridge, 2007).

I.1.6. Estresse Oxidativo

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

O rompimento entre o equilíbrio pró-oxidante e antioxidante é descrito como estresse oxidativo, e pode representar um mecanismo fundamental envolvido em doenças humanas (Halliwell e Gutteridge, 2007; Sies, 1985). O estresse oxidativo pode resultar tanto de uma diminuição das defesas antioxidantes quanto de uma produção aumentada de oxidantes, bem como da liberação de metais de transição ou a combinação de quaisquer desses fatores (Halliwell, 2001).

Como consequência, 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 ao dano 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 CAT e 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, 2007a).

I.1.7. Mecanismos de dano celular induzido por radicais livres

As ERO e ERN ocorrem tanto em processos fisiológicos quanto patológicos do organismo. Fisiologicamente, essas espécies reativas apresentam diversas funções (Bergendi *et al.*, 1999). Assim, um aumento da liberação local de radicais livres pode ser benéfico, como é o caso da liberação de espécies oxidantes pelos neutrófilos, que podem atuar na defesa do hospedeiro contra uma infecção (Delanty e Dichter, 1998). Participam ainda de processos de sinalização celular e também estão envolvidos na síntese e regulação de algumas proteínas (Son *et al.*, 2013).

Por outro lado, quando formadas em excesso, essas espécies altamente reativas têm o potencial de oxidar moléculas biológicas incluindo proteínas, lipídios e DNA (Sesti *et al*., 2012). Com relação aos efeitos prejudiciais das reações oxidantes ao organismo, os radicais livres podem promover lipoperoxidação e consequentemente a ruptura das membranas celulares; podem causar a oxidação de lipoproteínas de baixa densidade (LDL); podem reagir com proteínas, levando à sua inativação e consequente alteração de sua função; e podem também reagir com o DNA e RNA, levando a mutações somáticas e a distúrbios de transcrição (Delanty e Dichter, 1998), dentre outros efeitos.

I.1.7.1. Suscetibilidade cerebral ao dano oxidativo

O cérebro tem alta suceptibilidade ao dano oxidativo, quando comparado a outros tecidos como o fígado, devido às suas características e funções. Primeiro, por ter grande demanda energética para o seu funcionamento normal, o sistema nervoso central (SNC) tem a mitocôndria como uma estrutura intracelular fundamental. Dessa forma, visto que a produção eficiente de energia na forma de ATP pela mitocôndria é dependente de grandes quantidades de oxigênio, o alto consumo de O_2 se traduz em um aumento da atividade da cadeia respiratória e o consequente aumento na produção do radical superóxido pela dispersão dos elétrons.

Outra característica do SNC que contribui para a alta susceptibilidade ao dano oxidativo é o fato as membranas celulares serem ricas em ácidos graxos de cadeia lateral poliinsaturada (PUFA), os quais são especialmente sensíveis ao ataque oxidativo de radicais livres. Além disso, o alto conteúdo

de ferro, o qual favorece a lipoperoxidação e autooxidação de neurotransmissores, a grande quantidade de microglia e macrófagos residentes do sistema nervoso que podem produzir $O_{2^{\cdot-}}$ e H_2O_2 e a modesta defesa antioxidante (baixos níveis de catalase) aumentam a suscetibilidade do cérebro ao dano causado por radicais livres (Halliwell, 1996; Halliwell e Gutteridge, 2007a; Zaleska e Floyd, 1985).

I.2. OBJETIVOS

I.2.1. Objetivo geral

O presente trabalho teve como objetivo estudar importantes parâmetros de homeostase redox em várias estruturas cerebrais (córtex cerebral, estriado e hipocampo) e tecidos periféricos (fígado e coração) de camundongos *Gcdh*$^{-/-}$ e selvagens (*Gcdh*$^{+/+}$) submetidos a uma dieta normal ou a uma sobrecarga de Lis (injeção intraperitoneal ou dieta rica em Lis), visando uma melhor compreensão da fisiopatologia do dano tecidual na AG I.

I.2.2. Objetivos específicos

a) Avaliar parâmetros de estresse oxidativo, através de determinação do dano oxidativo lipídico (substâncias reativas ao ácido tiobarbitúrico - TBA-RS) e proteico (carbonilas e sulfidrilas), produção de espécies reativas (oxidação de 2',7'-diclorofluoresceína-diacetato – DCFH, nitratos e nitritos), defesas antioxidantes não enzimáticas (GSH) e enzimáticas (SOD, CAT, GPx, GR e G6PDH) em tecidos cerebrais (córtex cerebral, estriado e hipocampo) e periféricos (fígado e coração) de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ com 15, 30 e 60 dias de vida.

b) Avaliar o efeito de uma sobrecarga aguda de Lis (injeção intraperitoneal de 8 µmol/g de Lis) em tecidos cerebrais (córtex cerebral, estriado e hipocampo) e periféricos (fígado e coração) de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ com 15 e 30 dias de vida sobre os seguintes parâmetros de estresse oxidativo: TBA-RS, formação de carbonilas, conteúdo de sulfidrilas, oxidação de DCFH, formação de nitratos e nitritos, concentrações de GSH e atividades das

enzimas SOD, CAT, GPx, GR e G6PDH.

c) Investigar o efeito de uma sobrecarga dietética de Lis (2,8% e 4,7%) por 60 horas sobre os seguintes parâmetros de estresse oxidativo em tecidos cerebrais (córtex cerebral, estriado e hipocampo) e periféricos (fígado e coração) de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ com 30 dias de vida: TBA-RS, formação de carbonilas, conteúdo de sulfidrilas, oxidação de DCFH, formação de nitratos e nitritos, níveis de GSH e atividades das enzimas SOD, CAT, GPx, GR e G6PDH.

d) Investigar o efeito de uma sobrecarga de Lis na dieta (4,7%) por 60 horas sobre a viabilidade das células neuronais e astrocitárias, usando anticorpos para NeuN, Ki67, S100b e GFAP, bem como marcadores de estresse oxidativo, YNO2 e iNOS, em córtex cerebral e estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ com 30 dias de vida.

e) Investigar o efeito de uma sobrecarga de Lis na dieta (2,8%) por 40 dias (do 21º ao 60º dia de vida) sobre os seguintes parâmetros de estresse oxidativo em tecidos cerebrais (córtex cerebral e estriado) de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ com 30 dias de vida: TBA-RS, concentrações de GSH e atividades das enzimas SOD, CAT, GPx, GR e G6PDH .

g) Investigar o efeito de uma sobrecarga de Lis na dieta (2,8%) por 40 dias (do 21º ao 60º dia de vida) sobre a histologia do córtex cerebral e estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ com 60 dias de vida, avaliada pela coloração com hematoxilina e eosina.

PARTE II

Artigos Científicos

Capítulo I

Acute lysine overload provokes protein oxidative damage and reduction of antioxidant defenses in brain of infant glutaryl-CoA dehydrogenase deficient mice: a role for oxidative stress in GA I neuropathology

Bianca Seminotti, Rafael Teixeira Ribeiro, Alexandre Umpierrez Amaral, Carolina Coffi Pereira, Guilhian Leipnitz, David M. Koeller, Stephen Goodman, Michael Woontner, Moacir Wajner.

Artigo científico submetido para publicação no periódico

Journal of the Neurological Sciences

Acute lysine overload provokes protein oxidative damage and reduction of antioxidant defenses in brain of infant glutaryl-CoA dehydrogenase deficient mice: a role for oxidative stress in GA I neuropathology

Bianca Seminotti [a], Rafael Teixeira Ribeiro [a], Alexandre Umpierrez Amaral [a], Mateus Struecker da Rosa [a], Carolina Coffi Pereira [a], Guilhian Leipnitz [a], David M. Koeller [b], Stephen Goodman [c], Michael Woontner [c], Moacir Wajner [a,d*]

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

[b] Department of Pediatrics, Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR, USA.

[c] Department of Pediatrics, University of Colorado Denver, Aurora, CO, USA.

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

* Corresponding Author: Moacir Wajner

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

Abstract

We evaluated the antioxidant defense system and protein oxidative damage in brain and liver of 15-day-old GCDH deficient knock out (*Gcdh*$^{-/-}$) mice following an acute intraperitoneal administration of Lys (8 μmol/g). We determined reduced glutathione (GSH) concentrations, sulfhydryl content, carbonyl formation and the activities of the antioxidant enzymes glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) in brain and liver of these animals. 2',7'-Dichlorofluorescein (DCFH) oxidation was also measured as an index of free radical formation. The only parameters altered in *Gcdh*$^{-/-}$ compared to wild type (*Gcdh*$^{+/+}$) mice were a reduction of liver GSH concentrations and of brain sulfhydryl content. Acute Lys injection provoked a decrease of GSH concentration in the brain and sulfhydryl content in the liver, and an increase in carbonyl formation in brain and liver of *Gcdh*$^{-/-}$ mice. Lys administration also induced a decrease of all antioxidant enzyme activities in the brain and an increase of the activities of SOD and CAT in the liver of *Gcdh*$^{+/+}$ mice. Finally, Lys elicited a marked increase of DCFH oxidation in brain and liver. It is concluded that Lys overload compromises the brain antioxidant defenses and induces protein oxidation, probably secondary to reactive species generation, in infant *Gcdh*$^{+/+}$ mice.

Key words: glutaric acidemia type I, brain damage, antioxidant defenses, glutaric acid, 3-hydroxyglutaric acid, infant *Gcdh*$^{-/-}$ mice

Introduction

The mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) is responsible for the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA and CO_2 in the catabolic pathways of the amino acids lysine (Lys) and tryptophan [1]. Deficiency of GCDH activity is the biochemical defect of glutaric aciduria type I (GA I, McKusick 23167, OMIM #231670), a cerebral organic aciduria characterized by macrocephaly associated with frontotemporal atrophy at birth [2]. The biochemical hallmark of this disorder is the accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3OHGA) and glutarylcarnitine in tissues and biological fluids. Untreated patients normally develop acute encephalopathic crises between 6 months and 4 years of age that are frequently precipitated by catabolic states. These episodes lead to massive striatal degeneration and the appearance of neurological symptoms such as dystonia, dyskinesia, loss of motor control, seizures and coma [3]. Moreover, patients who do not suffer from acute episodes also develop chronic neurologic dysfunction [4-6]. Neuroradiologic findings include progressive cortical frontotemporal hypoplasia with white matter abnormalities (leukoencephalopathy), chronic subdural effusions, hematomas, as well as acute striatal degeneration with loss of medium spiny neurons and astrogliosis [2, 5, 7].

Several *in vitro* and *in vivo* experiments indicate that the accumulating organic acids GA and 3OHGA are related to the pathogenesis of this disorder, acting as neurotoxins in the brain, probably facilitating excitotoxicity, oxidative damage and impairment of cellular energy metabolism [8-30]. However, some results are contradictory, probably due to variation in the experimental approaches, and nearly all studies were performed in brain tissues with normal GCDH activity, which makes the pathophysiological relevance of these experiments uncertain.

Koeller and collaborators [31] developed a mouse knock out model of GA I, replacing much of the *GCDH* gene with an in-frame beta-galactosidase cassette. The animals showed a biochemical phenotype comparable to GA I patients, with accumulation of GA and 3OHGA and progressive leukodystrophy, although striatal degeneration was not observed, even under conditions of metabolic stress. Zinnanti and colleagues [32, 33] demonstrated that exposing these animals to high protein or lysine (Lys) intake resulted in striatal damage similar to the human condition. The neuropathological findings of these animals were comparable to human patients,

including neuronal loss and vacuolation, blood-brain barrier breakdown, myelin disruption and gliosis, mostly in the striatum and deep cortex.

In the present study we determined glutathione (GSH) concentrations, sulfhydryl content, carbonyl formation and the antioxidant enzyme activities glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) in brain and liver of wild type (*Gcdh*$^{+/+}$) and knockout (*Gcdh*$^{-/-}$) mice in order to evaluate the tissue antioxidant defenses and protein oxidation in a central (brain) and a peripheral (liver) tissue from these animals. We also studied the effects of an acute intraperitoneal (i.p.) injection of Lys in *Gcdh*$^{-/-}$ mice as an attempt to mimic the crises of metabolic decompensation that often appear to precede brain injury. 2',7'-Dichlorofluorescein (DCFH) oxidation, an index of reactive species generation, was also measured.

Material and Methods

Chemicals

All chemicals were of analytical grade and purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2-7.4 in the appropriate buffer for each technique.

Animals

Gcdh$^{+/+}$ and *Gcdh*$^{-/-}$ littermates, both of C129SvEv background, were generated from heterozygotes and maintained at Unidade Experimental Animal of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). 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 ºC) colony room, with free access to water and 20 % (w/w) protein commercial chow containing 0.9 % Lys (SUPRA, Porto Alegre, RS, Brazil). Fifteen-day-old male *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice were used in all experiments, corresponding to humans 3 to 4 years of age.

Ethical statement

This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America,

NIH, publication n 85-23, revised in 2011, and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Hospital de Clínicas de Porto Alegre. All efforts were made to minimize suffering, discomfort, stress and the number of animals necessary to produce reliable scientific data.

Lys administration

A group of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ animals were given a single i.p. injection of Lys (8 μmol/g) or saline. The parameters of oxidative stress were measured 24 hours after Lys injection.

Tissue Preparation

The mice were anesthetized with a mixture of ketamine (90 mg/kg) and xilazine (10 mg/kg) and transcardiacally perfused for 5 min with saline solution. After perfusion, brain and liver were rapidly removed. The tissues were homogenized in 9 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 pellet nuclei and cell debris [34]. The pellet was discarded and the supernatant, which included mitochondria, was retained for the measurements. Tissue slices (400 μm) were also prepared from the brain and liver for DCFH oxidation determination.

Reduced glutathione (GSH) levels

GSH levels were measured according to Browne and Armstrong [35]. One volume of metaphosphoric acid was added to 150 mL of tissue supernatants, which were centrifuged for 10 min at 7000 *g*. Then 30 mL from the pre-treated supernatants were diluted with 70 mL of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. This preparation was incubated with o-phthaldialdehyde (1 mg/mL in methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Concentrations were calculated from a calibration curve of a GSH standard (0.001–1mM) and expressed as nmol/mg protein.

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 [36]. Briefly, 30 µL of 10 mM DTNB and 980 µL of PBS were added to 50 µL of tissue supernatants containing 0.3 mg of protein. This was followed by a 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were calculated and expressed as nmol/mg protein.

Protein carbonyl formation

Protein carbonyl formation, a marker of protein oxidative damage, was measured spectrophotometrically according to Reznick and Packer [37]. Two hundred microliters of tissues supernatants were treated with 400 µL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl alone (blank) and left in the dark for 1 h. Samples were then precipitated with 600 mL 20 % trichloroacetic acid 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 mL of 6 M guanidine prepared in 2.5 N HCl at 37 ^{o}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 and expressed as nmol of carbonyl groups/mg of protein, using the extinction coefficient of 22,000 × 10^6 nmol/mL for aliphatic hydrazones [38].

Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel [39] 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 / 1 mM ethylenediaminetetraacetic acid, pH 7.7, 2 mM GSH, 0.1 U / mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH and tissue supernatants (approximately 3 µg of protein). One GPx unit (U) is defined as 1 µmol of NADPH consumed per minute. The specific activity was calculated and expressed as U / mg protein.

Superoxide dismutase (SOD) activity

SOD activity was assayed according to Marklund [40] and is based on the capacity of pyrogallol to autooxidize, a process highly dependent on $O_2^{\bullet-}$, which is a

substrate for SOD. The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer / 1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U / mL catalase, 0.38 mM pyrogallol and tissue supernatants (approximately 1 μg of protein). A calibration curve was performed with purified SOD as standard to calculate the activity of SOD present in the samples. The results were calculated and expressed as U / mg protein.

Catalase (CAT) activity

CAT activity was assayed according to Aebi [41] 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 tissue supernatants (approximately 1 μg of protein). One unit (U) of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute. The specific activity was calculated and expressed as U / mg protein.

Glutathione reductase (GR) activity

GR activity was measured according to Calberg and Mannervik [42] using oxidized glutathione (GSSG) and NADPH as substrates. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium with 200 mM sodium phosphate buffer, pH 7.5, containing 6.3 mM ethylenediaminetetraacetic acid, 1 mM GSSG, 0.1 mM NADPH and tissue supernatants (approximately 3 μg of protein). One GR unit (U) is defined as 1 μmol of GSSG reduced per minute. The specific activity was calculated and expressed as U / mg protein.

2',7'-Dihydrodichlorofluorescein (DCFH) oxidation

Reactive species production was assessed according to LeBel et al [43] by using 2',7'-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA was prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl and incubated with tissue slices (30 mg) during 30 min at 37 ^{o}C. DCF-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of reactive species. The DCF fluorescence intensity parallels the amount of

reactive species present. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. A calibration curve was prepared with DCF standards (0.25-10 mM) and the levels of reactive species were calculated from the curve and expressed as pmol DCF formed / mg protein.

Protein Determination

Protein content was measured by the method of Lowry et al [44] using bovine serum albumin as standard.

Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Data were analysed 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 considered 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.

Results

Reduced brain and liver GSH concentrations in Gcdh$^{-/-}$ *mice*

First, we observed that GSH levels were similar in the brain (Figure 1A), but markedly diminished (up to 80%) in the liver of *Gcdh*$^{-/-}$, as compared to *Gcdh*$^{+/+}$ mice [$F_{(3,15)} = 47.666$, $P < 0.001$] (Figure 1B). Lys administration decreased GSH concentrations (up to 15%) in the brain [$F_{(3,15)} = 2.595$, $P < 0.05$] (Figure 1A), but did not further reduce these concentrations in the liver (Figure 1B).

Reduced brain and liver sulfhydryl content in Gcdh$^{-/-}$ *mice*

Next, we showed that sulfhydryl content was markedly reduced in the brain (up to 45%) (Figure 2A) [$F_{(3,15)} = 42.699$, $P < 0.001$], but not in the liver (Figure 2B) of *Gcdh*$^{-/-}$, as compared to *Gcdh*$^{+/+}$, mice. Sulfhydryl content was not further diminished in the brain of *Gcdh*$^{-/-}$ mice after Lys administration. However, a single Lys injection did reduce sulfhydryl content in the liver of these knockout mice [$F_{(3,12)} = 6.307$, $P < 0.01$] (up to 25%) (Figure 2B).

Lys administration increases brain and liver protein carbonyl formation in* Gcdh$^{-/-}$ *mice

Figure 3 shows that carbonyl formation was not different in *Gcdh*$^{-/-}$ mice, as compared to *Gcdh*$^{+/+}$ mice. Lys injection provoked a statistically significant increase of carbonyl formation in the brain (up to 15%) [$F_{(3,15)} = 4.873$, $P < 0.05$] (Figure 3A) and liver (up to 14%) [$F_{(3,12)} = 3.198$, $P < 0.05$] (Figure 3B) of *Gcdh*$^{-/-}$ mice.

Lys administration alters brain and liver enzymatic antioxidant defenses in* Gcdh$^{-/-}$ *mice

In the brain, we observed that the activities of the antioxidant enzymes GPx, CAT and GR were similar in *Gcdh*$^{-/-}$ and *Gcdh*$^{+/+}$ mice (Figures 4 and 5). However, there was a statistically significant decrease of all these enzyme activities 24 hours after Lys [GPx: $F_{(3,12)} = 13.487$, $P < 0.001$, up to 50%; SOD: $F_{(3,12)} = 10.320$, $P < 0.01$, up to 70%; CAT: $F_{(3,12)} = 17.542$, $P < 0.05$, up to 33%; GR: $F_{(3,12)} = 15.331$, $P < 0.05$, up to 15%] (Figure 4). Interestingly, we found a marked increase of SOD (up to 65%) [$F_{(3,12)} = 11.898$, $P < 0.01$] and CAT (up to 25%) [$F_{(3,12)} = 28.655$, $P < 0.001$] activities in the liver of *Gcdh*$^{-/-}$ mice that received Lys, with no alterations of GPx and GR activities (Figure 5).

Lys administration increases brain and liver reactive species generation (DCFH oxidation) in* Gcdh$^{-/-}$ *mice

We also found that although DCFH oxidation did not differ between *Gcdh*$^{-/-}$ and *Gcdh*$^{+/+}$ mice, Lys increased DCFH oxidation in both the brain (up to 35%) [$F_{(3,12)} = 9.023$, $P < 0.001$] (Figure 6A) and liver (up to 25%) [$F_{(3,14)} = 11.001$, $P < 0.01$] (Figure 6B).

Discussion

Although the exact molecular mechanisms involved in GA I pathophysiology are still poorly understood, it seems that GA and 3OHGA, the major Lys-derived accumulating metabolites, play a decisive role in the brain damage characteristic of this disease [29]. In line with this assumption, Lys dietary restriction is currently used to prevent striatal degeneration in affected patients, implying the need to decrease brain GA and 3OHGA concentrations [15, 33, 45, 46].

Astrogliosis, which may be associated with increased production of reactive oxygen species [48, 49, 50], was observed in the striatal and cortical abnormalities of *Gcdh*$^{-/-}$ mice treated with high dietary Lys [33]. Recent studies from our laboratory showed that Lys administration induced oxidative stress *in vivo* in the brains of adult *Gcdh*$^{-/-}$ mice [50, 51], but the redox homeostasis in younger (infant or adolescent) animals has not been yet investigated. Therefore, in the present work we evaluated the antioxidant defenses and protein oxidative damage in a central (brain) and a peripheral (liver) tissue from infant (15-old-day) *Gcdh*$^{-/-}$ mice. We also investigated the effects of a single Lys acute overload (8 μmol/g) in these animals as an attempt to mimic a situation of acute metabolic decompensation. 15-day-old mice correspond to 2-4-year-old humans, an age in which GA I patients are vulnerable to acute degeneration [15, 52, 53].

The results obtained in the present investigation with infant mice showed that GSH concentrations and sulfhydryl content were reduced in liver and brain, respectively, from *Gcdh*$^{-/-}$, as compared to *Gcdh*$^{+/+}$ mice under a normal chow that contains 0.9 % Lys. Furthermore, Lys acute injection provoked marked alterations of the antioxidant defenses in the brain and liver of *Gcdh*$^{-/-}$ mice and to a higher extent than those we previously reported with adult mice [46]. It has been shown previously that serum and brain Lys levels do not change in *Gcdh*$^{+/+}$ and symptomatic and asymptomatic *Gcdh*$^{-/-}$ mice submitted to a high Lys overload [32, 33], making it unlikely that brain Lys accumulation could be responsible for the disruption of redox homeostasis observed in the present and previous studies. Because acute and chronic Lys administration provokes an increase of GA in the brain of *Gcdh*$^{-/-}$ mice [33, 50], it is concluded that the altered oxidative stress parameters were mainly due to the high levels of this organic acid in the brain. This is consistent with previous observations

showing that GA and 3OHGA alter the redox homeostasis *in vitro* and *in vivo* in brain of rodents [23-28].

The reduction of the concentrations of GSH, one of the most important naturally occurring antioxidants, in liver and brain from *Gcdh*$^{-/-}$ mice receiving Lys injection indicate a compromised endogenous antioxidant defense system. Sulfydryl content was also reduced, especially in brain, of Lys-supplemented *Gcdh*$^{-/-}$mice. Since approximately two thirds of sulfhydryl groups are bound to proteins [54], it is presumed that reduced sulfhydryl content reflects oxidation of cellular proteins that may potentially lead to inactivation [55, 56]. Carbonyl formation, a marker of free radical-mediated protein oxidation [57], was also increased in the brain and liver after Lys administration. It is therefore assumed that induction of protein oxidative damage occurred in brain and liver of the Lys-treated *Gcdh*$^{-/-}$ mice.

In order to further evaluate the antioxidant defense system, we measured various antioxidant enzyme activities and observed that these activities were not changed in brain and liver from *Gcdh*$^{-/-}$ mice fed a normal chow. However, Lys overload elicited reductions of GPx, DAT, SOD and GR enzyme activities in the brain, whereas SOD and CAT activities were increased in the liver. The distinct responses of brain and liver to the high GA levels induced by Lys are probably due to the fact that the liver has a much higher regenerative capacity and adaptability to metabolic alterations regulating body homeostasis than the brain [58, 59, 60]. The increased SOD and CAT activities observed in liver from from *Gcdh*$^{-/-}$ mice that received Lys injection may be due to an up regulation of these antioxidant enzyme genes secondary to superoxide and hydrogen peroxide overproduction. On the contrary, the central nervous system is considered to be particularly vulnerable to oxidative damage due to its high metabolic rate coupled to reactive oxygen species, high levels of polyunsaturated fatty acids and the comparatively low content of antioxidant systems, implying a lower capacity to react against free radicals compared with other tissues [61, 62]. Therefore, it is presumed that the decrease of the antioxidant enzyme activities verified in brain was probably a consequence of a direct attack of reactive species to site-specific amino acids in enzyme structure as previously shown in other pathological states [63, 64, 65].

Our previous work carried out in 30-day-old *Gcdh*$^{-/-}$ mice revealed that acute and chronic Lys overload provoked alterations of these antioxidant activities only in the brain [50, 51]. The younger mice tested here appear to have an even greater

vulnerability to oxidative damage. The impairment of the antioxidant system and the induction of protein oxidative damage in brain and liver of the *Gcdh*$^{-/-}$ mice may be a result of the generation of reactive species in this animal model of GA I, since Lys administration caused increased DCFH oxidation, which is a probe of ROS. The present results are in line with the current therapeutic procedure of restricting Lys dietary intake to GA I patients, especially in the first few years of life, where the brain is highly vulnerable to degeneration [15, 45].

In conclusion, we have provided experimental evidence for a disruption of the antioxidant capacity and induction of protein oxidative damage, especially in brain, of infant *Gcdh*$^{-/-}$ mice treated with a single Lys overload. Since Lys can rapidly penetrate into the brain where it is mainly converted to GA and also to 3OHGA [33, 47] and since GA and also to 3OHGA were shown to induce *in vivo* and *in vitro* oxidative stress in brain of rodents [23, 25, 26, 28, 30], we postulate that our results are due to brain accumulation of these organic acids. Finally, our findings suggest that antioxidants, especially those targeted to the mitochondria [66, 67], may be adjuvant therapies together with Lys dietary restriction and L-carnitine supplementation in the treatment of GA I patients, especially in the first years of life and/or during encephalopathic crises when the levels of the accumulating metabolites dramatically increase.

Acknowlegments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Programa de Apoio a Núcleos de Excelência (PRONEX), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Pró-Reitoria de Pesquisa / Universidade Federal do Rio Grande do Sul (PROPESQ/UFRGS), Financiadora de estudos e projetos (FINEP), Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00 and Instituto Nacional de Ciência e Tecnologia em Excitotoxicidade e Neuroproteção (INCT-EN).

Legends for Figures

Fig. 1. Glutathione (GSH) concentrations in brain (A) and liver (B) of *Gdch*$^{+/+}$ and *Gcdh*$^{-/-}$ mice. GSH concentrations were also measured 24 h after a single injection of

saline or lysine (Lys; 8 μmol/g) administered intraperitoneally to *Gcdh*$^{-/-}$ mice. Data are represented as mean ± SD for 4 to 5 independent experiments (animals). *P < 0.05, compared to *Gcdh*$^{-/-}$ mice injected with saline; $^{\#}P < 0.05$ $^{\#\#\#}P < 0.001$, compared to *Gcdh*$^{+/+}$ mice (Duncan multiple range test).

Fig. 2. Sulfhydryl content in brain (A) and liver (B) of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice. Sulfhydryl content was also measured 24 h after a single injection of saline or lysine (Lys; 8 μmol/g) administered intraperitoneally to *Gcdh*$^{-/-}$ mice. Data are represented as mean ± SD for 5 independent experiments (animals). *P < 0.05, compared to *Gcdh*$^{-/-}$ mice injected with saline; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to *Gcdh*$^{+/+}$ mice (Duncan multiple range test).

Fig. 3. Carbonyl formation in brain (A) and liver (B) of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice 24h after saline or lysine injection. Carbonyl formation was also measured 24 h after a single injection of saline or lysine (Lys; 8 μmol/g) administered intraperitoneally to *Gcdh*$^{-/-}$ mice. Data are represented as mean ± SD for 5 independent experiments (animals). ***P < 0.001, compared to *Gcdh*$^{-/-}$ mice; $^{\#\#}P < 0.01$, compared to *Gcdh*$^{+/+}$ mice (Duncan multiple range test).

Fig. 4. Glutathione peroxidase (GPx) (A), superoxide dismutase (SOD) (B), catalase (CAT) (C) and glutathione reductase (GR) (D) activities in brain of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice. The enzyme activities were also measured 24 h after a single injection of saline or lysine (Lys; 8 μmol/g) administered intraperitoneally to *Gcdh*$^{-/-}$ mice. Data are represented as mean ± SD for 5 independent experiments (animals). *P <

0.05, ***$P < 0.001$, compared to *Gcdh*$^{-/-}$ mice injected with saline; $^{\#}P < 0.05$, $^{\#\#\#}P <$ 0.001, compared to *Gcdh*$^{+/+}$ mice (Duncan multiple range test).

Fig. 5. Glutathione peroxidase (GPx) (A), superoxide dismutase (SOD) (B), catalase (CAT) (C) and glutathione reductase (GR) (D) activities in liver of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice. The enzyme activities were also measured 24 h after a single injection of saline or lysine (Lys; 8 μmol/g) administered intraperitoneally to *Gcdh*$^{-/-}$ mice. Data are represented as mean ± SD for 5 independent experiments (animals). ***$P <$ 0.001, compared to *Gcdh*$^{-/-}$ mice injected with saline; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to *Gcdh*$^{+/+}$ mice (Duncan multiple range test).

Fig. 6. 2',7'-Dihydrodichlorofluorescein (DCFH) oxidation in brain (A) and liver (B) of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice. DCFH oxidation was also measured 24 h after a single injection of saline or lysine (Lys; 8 μmol/g) administered intraperitoneally to *Gcdh*$^{-/-}$ mice. Data are represented as mean ± SD for 5 independent experiments (animals). **$P < 0.01$, ***$P < 0.001$, compared to *Gcdh*$^{-/-}$ mice injected with saline; $^{\#\#}P < 0.01$, compared to *Gcdh*$^{+/+}$ mice (Duncan multiple range test).

Figures
Fig. 1

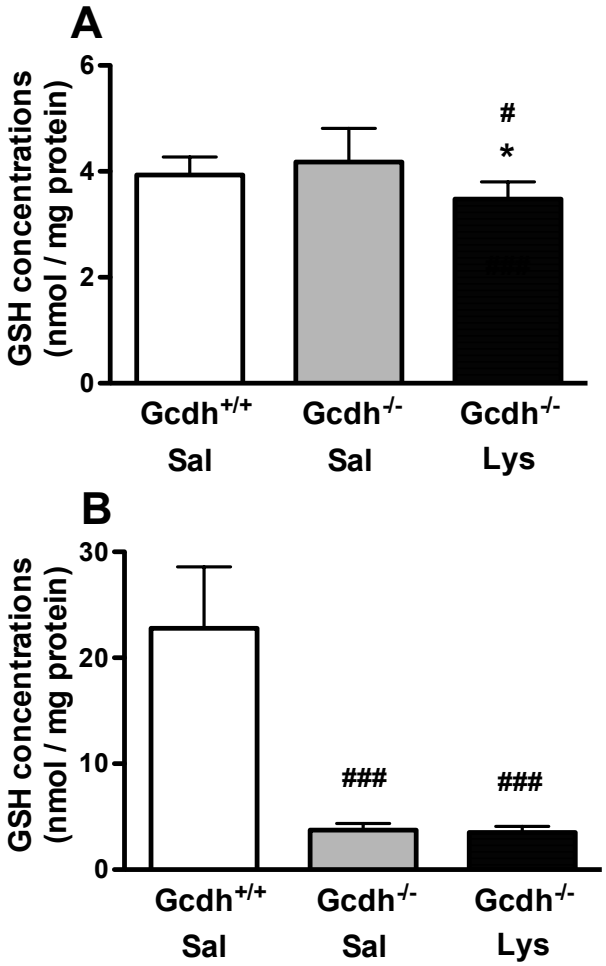


Fig. 2

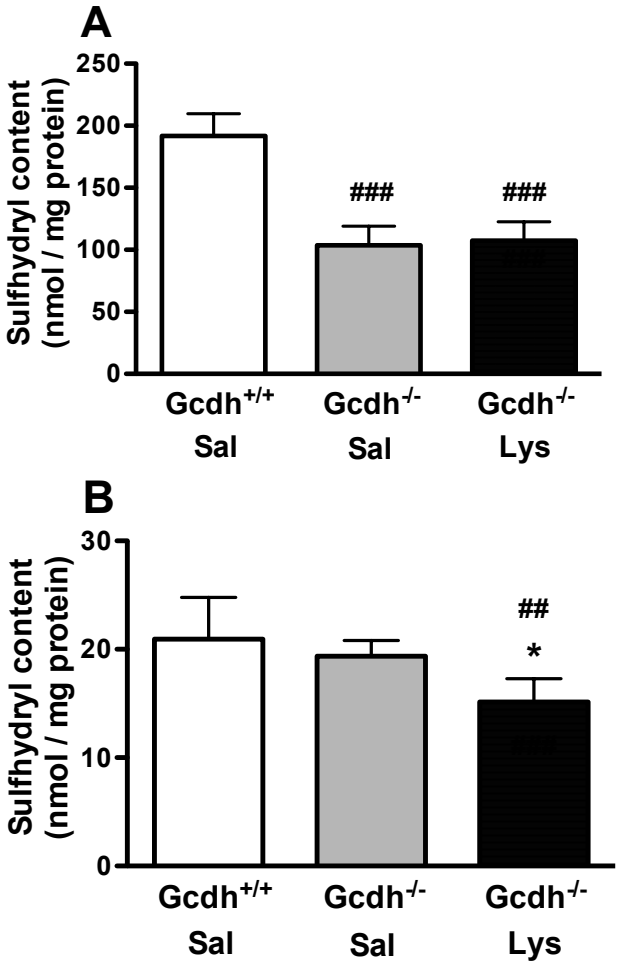

A
Sulfhydryl content (nmol / mg protein)
250
200
150
100
50
0
###
###
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys
B
Sulfhydryl content (nmol / mg protein)
30
20
10
0
##
*
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys

Fig. 3

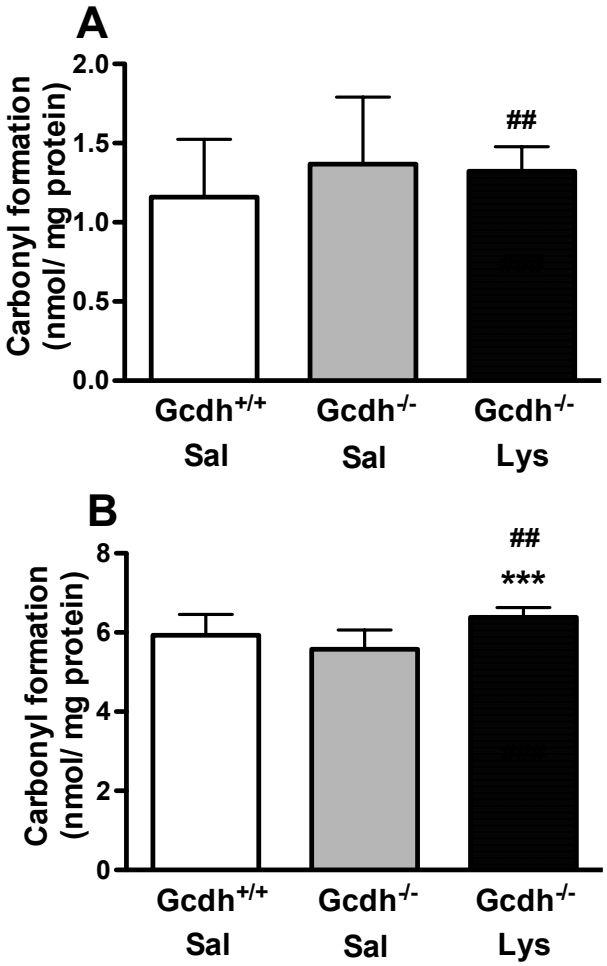

A
Carbonyl formation (nmol/ mg protein)
2.0
1.5
1.0
0.5
0.0
##
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys
B
Carbonyl formation (nmol/ mg protein)
8
6
4
2
0
##
***
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys

Fig. 4

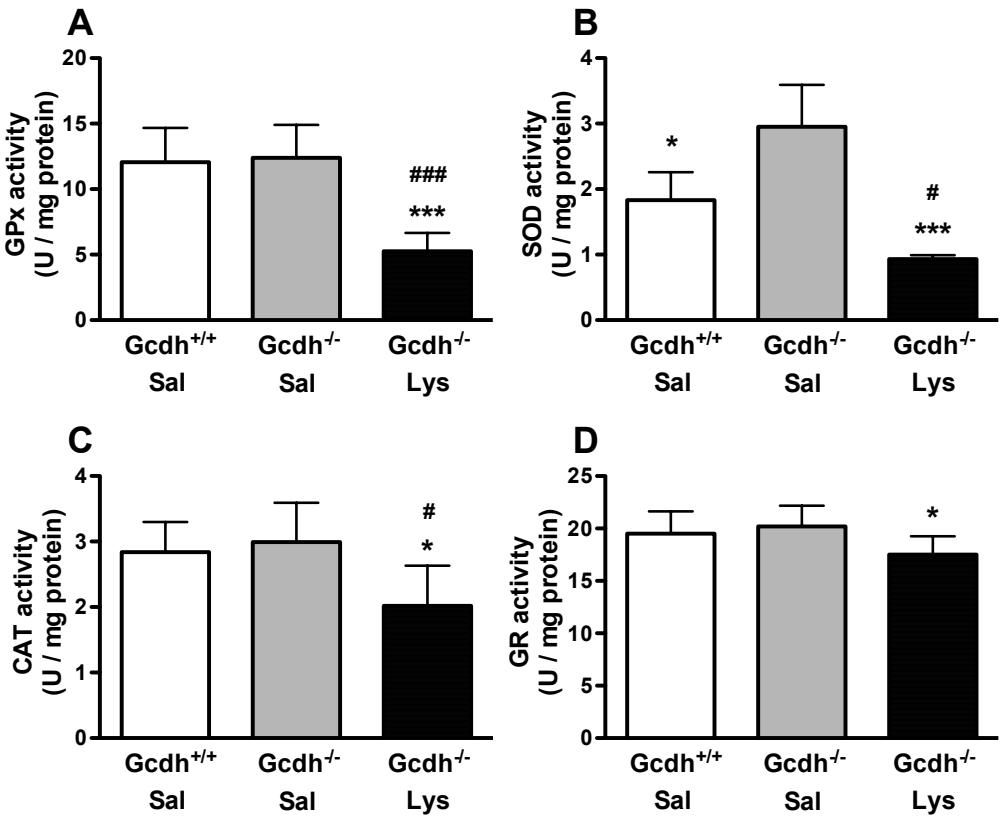

A
GPx activity
(U / mg protein)
20
15
10
5
0
###
***
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys
B
SOD activity
(U / mg protein)
4
3
2
1
0
*
#
***
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys
C
CAT activity
(U / mg protein)
4
3
2
1
0
#
*
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys
D
GR activity
(U / mg protein)
25
20
15
10
5
0
*
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys

Fig. 5

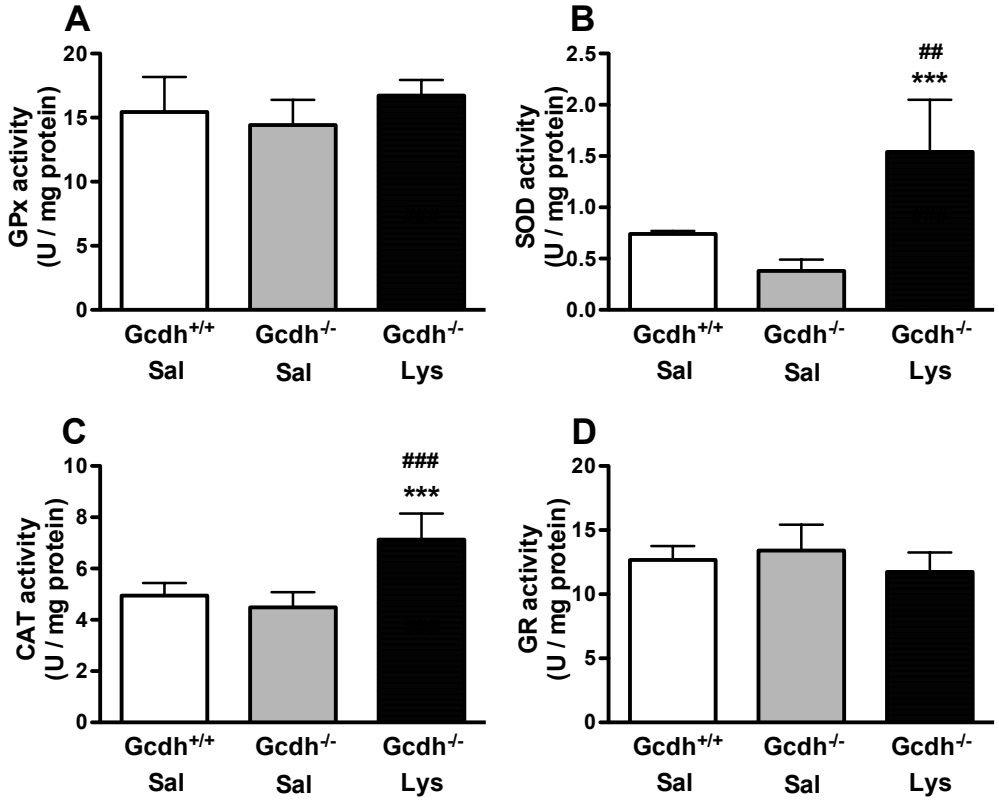
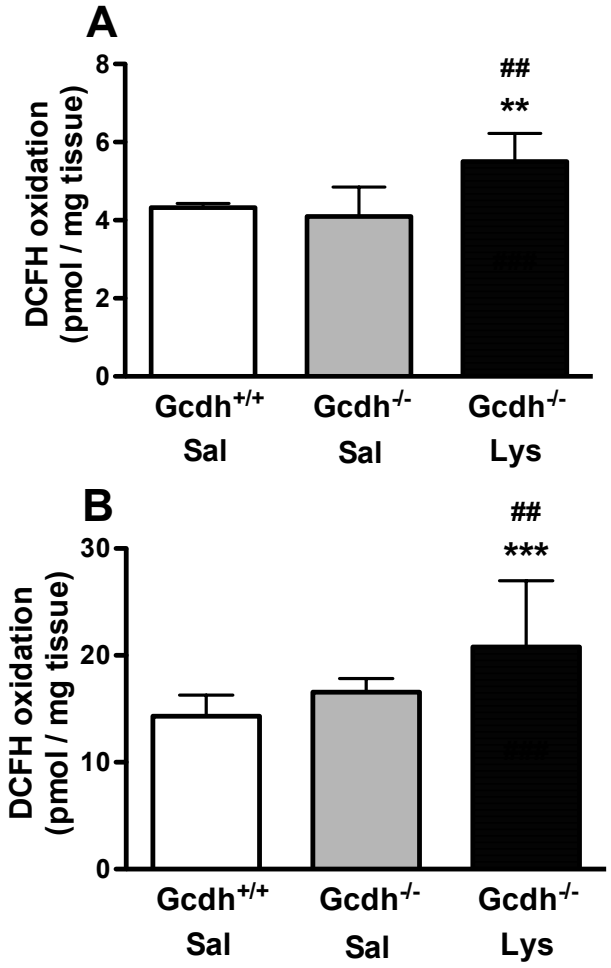

A
GPx activity (U / mg protein)
20
15
10
5
0
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys
B
SOD activity (U / mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
##
***
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys
C
CAT activity (U / mg protein)
10
8
6
4
2
0
###
***
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys
D
GR activity (U / mg protein)
20
15
10
5
0
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys

Fig. 6


A
DCFH oxidation (pmol / mg tissue)
0
2
4
6
8
##
**
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys
B
DCFH oxidation (pmol / mg tissue)
0
10
20
30
##
***
Gcdh+/+ Sal
Gcdh-/- Sal
Gcdh-/- Lys

References

[1] Christensen E. A fibroblast glutaryl-CoA dehydrogenase assay using detritiation of 3H-labelled glutaryl-CoA: application in the genotyping of the glutaryl-CoA dehydrogenase locus. Clin Chim Acta 1993;220:71-80.

[2] Goodman SI, Norenberg MD, Shikes RH, Breslich DJ, Moe PG. Glutaric aciduria: biochemical and morphologic considerations. J Pediatr 1977;90: 746-750.

[3] Strauss KA, Puffenberger EG, Robinson DL, Morton DH. Type I glutaric aciduria, part 1: natural history of 77 patients. Am J Med Genet C Semin Med Genet 2003;121C: 38-52.

[4] Strauss KA, Lazovic J, Wintermark M, Morton DH. Multimodal imaging of striatal degeneration in Amish patients with glutaryl-CoA dehydrogenase deficiency. Brain 2007;130:1905-1920.

[5] Harting I, Neumaier-Probst E, Seitz A *et al*. Dynamic changes of striatal and extrastriatal abnormalities in glutaric aciduria type I. Brain 2009;132:1764-1782.

[6] Heringer J, Boy SP, Ensenauer R. Use of guidelines improves the neurological outcome in glutaric aciduria type I. Ann Neurol 2010;68:743-752.

[7] Kimura S, Hara M, Nezu A, Osaka H, Yamazaki S, Saitoh K. Two cases of glutaric aciduria type 1: clinical and neuropathological findings. J Neurol Sci 1994;123: 38-43.

[8] Stokke O, Goodman SI, Moe PG. Inhibition of brain glutamate decarboxylase by glutarate, glutaconate, and beta-hydroxyglutarate: explanation of the symptoms in glutaric aciduria? Clin Chim Acta 1976;66: 411-415.

[9] Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF. 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors in vitro. J Inherit Metab Dis 1999;22: 259-262.

[10] Kölker S, Ahlemeyer B, Krieglstein J, Hoffmann GF. Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr Res 2001;50: 76-82.

[11] Kölker S, Ahlemeyer B, Hühne R, Mayatepek E, Krieglstein J, Hoffmann GF. Potentiation of 3-hydroxyglutarate neurotoxicity following induction of astrocytic iNOS in neonatal rat hippocampal cultures. Eur J Neurosci 2001;13: 2115-2122.

[12] Kölker S, Okun JG, Ahlemeyer B et al. Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons. J Neurosci Res 2002;68: 424-431.

[13] Kölker S, Köhr G, Ahlemeyer B et al. Ca(2+) and Na(+) dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr Res 2002;52:199-206.

[14] Kölker S, Koeller DM, Sauer S et al. Excitotoxicity and bioenergetics in glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 2004;27:805-812.

[15] Kölker S, Sauer SW, Okun JG, Hoffmann GF, Koeller DM. Lysine intake and neurotoxicity in glutaric aciduria type I: towards a rationale for therapy? Brain 2006;129: e54.

[16] Porciúncula LO, Dal-Pizzol A Jr, Coitinho AS, Emanuelli T, Souza DO, Wajner M. Inhibition of synaptosomal [3H]glutamate uptake and [3H]glutamate binding to plasma membranes from brain of young rats by glutaric acid in vitro. J Neurol Sci 2000;173: 93-96.

[17] Porciúncula LO, Emanuelli T, Tavares RG et al. Glutaric acid stimulates glutamate binding and astrocytic uptake and inhibits vesicular glutamate uptake in forebrain from young rats. Neurochem Int 2004;45:1075-1086.

[18] de Oliveira Marques F, Hagen ME, Pederzolli CD et al. Glutaric acid induces oxidative stress in brain of young rats. Brain Res 2003;964:153-158.

[19] Rosa RB, Schwarzbold C, Dalcin KB et al. Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats. Neurochem Int 2004;45:1087-1094.

[20] Rosa RB, Dalcin KB, Schmidt AL et al. Evidence that glutaric acid reduces glutamate uptake by cerebral cortex of infant rats. Life Sci 2007;81: 1668-1676.

[21] Wajner M, Kölker S, Souza DO, Hoffmann GF, de Mello CF. Modulation of glutamatergic and GABAergic neurotransmission in glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 2004;27:825-828.

[22] Dalcin KB, Rosa RB, Schmidt AL et al. Age and brain structural related effects of glutaric and 3-hydroxyglutaric acids on glutamate binding to plasma membranes during rat brain development. Cell Mol Neurobiol 2007;27:805-818.

[23] Fighera MR, Royes LF, Furian AF et al. GM1 ganglioside prevents seizures, Na+,K+-ATPase activity inhibition and oxidative stress induced by glutaric acid and pentylenetetrazole. Neurobiol Dis 2006;22:611-623.

[24] Latini A, Borba Rosa R, Scussiato K, Llesuy S, Belló-Klein A, Wajner M. 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Res 2002;956:367-373.

[25] Latini A, Scussiato K, Leipnitz G, Dutra-Filho CS, Wajner M. Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum. J Inherit Metab Dis 2005;28:57-67.

[26] Latini A, Ferreira GC, Scussiato K et al. Induction of oxidative stress by chronic and acute glutaric acid administration to rats. Cell Mol Neurobiol 2007;27:423-438.

[27] Olivera S, Fernandez A, Latini A et al. Astrocytic proliferation and mitochondrial dysfunction induced by accumulated glutaric acidemia I (GAI) metabolites: possible implications for GAI pathogenesis. Neurobiol Dis 2008;32:528-534.

[28] Magni DV, Furian AF, Oliveira MS et al. Kinetic characterization of l-[(3)H]glutamate uptake inhibition and increase oxidative damage induced by glutaric acid in striatal synaptosomes of rats. Int J Dev Neurosci 2009;27:65-72.

[29] Jafari P, Braissant O, Bonafé L, Ballhausen D. The unsolved puzzle of neuropathogenesis in glutaric aciduria type I. Mol Genet Metab 2011; 104:425-37.

[30] Olivera-Bravo S, Fernández A, Sarlabós MN et al. Neonatal astrocyte damage is sufficient to trigger progressive striatal degeneration in a rat model of glutaric acidemia-I. PLoS One 2011;6: e20831.

[31] Koeller DM, Woontner M, Crnic LS et al. Biochemical, pathologic and behavioral analysis of a mouse model of glutaric acidemia type I. Hum Mol Genet 2002;11:347-357.

[32] Zinnanti WJ, Lazovic J, Wolpert EB et al. A diet-induced mouse model for glutaric aciduria type I. Brain 2006;129:899-910.

[33] Zinnanti WJ, Lazovic J, Housman C. Mechanism of age-dependent susceptibility and novel treatment strategy in glutaric acidemia type I. J Clin Invest 2007;117:3258-3270.

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

[35] Browne RW, Armstrong D. Reduced glutathione and glutathione disulfide. Methods Mol Biol 1998;108:347-352.

[36] Aksenov MY, Markesbery WR. Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 2001;302:141-145.

[37] Reznick AZ, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol 1994;233:357-63.

[38] Levine RL, Garland D, Oliver CN et al. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990;186:464-78.

[39] Wendel A. Glutathione peroxidase. Meth Enzymol 1981;77:325–332.

[40] Marklund SL. Pyrogallol autoxidation. In Handbook for oxygen radical research, Boca Raton, FL: CRC Press, 1985:243–247.

[41] Aebi H. Catalase in vitro. Method Enzymol 1984;105:121–126.

[42] Calberg I, Mannervik B. Glutathione Reductase. Method Enzymol 1985;113:484-490.

[43] LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 1992;5:227-231.

[44] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-275.

[45] Strauss KA, Morton DH. Type I glutaric aciduria, part 2: a model of acute striatal necrosis. Am J Med Genet C Semin Med Genet 2003;121C:53-70.

[46] Strauss KA, Brumbaugh J, Duffy A et al. Safety, efficacy and physiological actions of a lysine-free, arginine-rich formula to treat glutaryl-CoA dehydrogenase deficiency: Focus on cerebral amino acid influx. Mol Genet Metab 2011;104:93-106.

[47] Qin L, Li G, Qian X et al. Interactive role of the toll-like receptor 4 and reactive oxygen species in LPS-induced microglia activation. Glia 2005;52:78-84.

[48] Levesque S, Wilson B, Gregoria V et al. Reactive microgliosis: extracellular μ-calpain and microglia-mediated dopaminergic neurotoxicity. Brain 2010;133:808–821.

[49] Sarafian TA, Montes C, Imura T et al. Disruption of astrocyte STAT3 signaling decreases mitochondrial function and increases oxidative stress In vitro. PloS One 2010;5:e9532.

[50] Seminotti B, da Rosa MS, Fernandes CG et al. Induction of oxidative stress in brain of glutaryl-CoA dehydrogenase deficient mice by acute lysine administration. Mol Genet Metab 2012;106:31-8.

[51] Seminotti B, Amaral AU, da Rosa MS et al. Disruption of brain redox homeostasis in glutaryl-CoA dehydrogenase deficient mice treated with high dietary lysine supplementation. Mol Genet Metab 2013;108:30-9.

[52] Goodman S. Frerman F. Organic acidemias due to defects in lysine oxidation: 2-ketoadipic acidemia and glutaric acidemia. C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill Inc., New York 2001;2195–2204

[53] Sauer SW, Okun JG, Fricker G et al. 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 2006;97:899-910.

[54] Requejo R, Hurd TR, Costa NJ, Murphy MP. Cysteine residues exposed on protein surfaces are the dominant intramitochondrial thiol and may protect against oxidative damage. FEBS J 2010;277:1465-80.

[55] Aksenov MY, Markesbery WR. Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 2001;302:141-5.

[56] Davies MJ. Singlet oxygen-mediated damage to proteins and its consequences. Biochem Biophys Res Commun 2003;305:761-70.

[57] Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 1994;233:346-57.

[58] Hallbergson AF, Gnatenco C, Peterson DA. Neurogenesis and brain injury: managing a renewable resource for repair. J Clin Invest. 2003 112:1128-33.

[59] Santos MS, Santos DL, Palmeira CM, Seiça R, Moreno AJ, Oliveira CR. Brain and liver mitochondria isolated from diabetic Goto-Kakizaki rats show different susceptibility to induced oxidative stress. Diabetes Metab Res Rev 2001;17:223-30.

[60] Tarlá MR, Ramalho F, Ramalho LN et al. Cellular aspects of liver regeneration. Acta Cir Bras 2006;21:63-6.

[61] Halliwell B, Gutteridge JMC. Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. In Halliwell B, Gutteridge JMC, eds. Free radicals in biology and medicine. Oxford: Oxford University Press Inc 2007;187–340.

[62] Halliwell B, Gutteridge JM. The importance of free radicals and catalytic metal ions in human diseases. Mol Aspects Med 1985;8:89-193.

[63] Brown GC. Nitric oxide and neuronal death. Nitric Oxide 2010;23:153–165.

[64] Singh P, Jain A, Kaur G. Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage. Mol. Cell. Biochem 2004;260:153–159.

[65] de Cavanagh EM, Inserra F, Ferder L, Fraga CG. Enalapril and captopril enhance glutathione-dependent antioxidant defenses in mouse tissues. Am J Physiol Regul Integr Comp Physiol 2000;278:R572-7.

[66] Cortés-Rojo C, Rodríguez-Orozco AR. Importance of oxidative damage on the electron transport chain for the rational use of mitochondria-targeted antioxidants. Mini Rev Med Chem 2011;11:625-632.

[67] Min K, Smuder AJ, Kwon OS, Kavazis AN, Szeto HH, Powers SK. Mitochondrial-targeted antioxidants protect the skeletal muscle against immobilization-induced muscle atrophy. J Appl Physiol Aug 4 2011;111:1459-66.

Capítulo II

Induction of oxidative stress in brain of glutaryl-CoA dehydrogenase deficient mice by acute lysine administration

Bianca Seminotti, Mateus Struecker da Rosa, Carolina Gonçalves Fernandes, Alexandre Umpierrez Amaral, Luisa Macedo Braga, Guilhian Leipnitz, Diogo Onofre Gomes de Souza, Michael Woontner, David M. Koeller, Stephen Goodman, Moacir Wajner.

Artigo científico publicado no periódico

Molecular Genetics and Metabolism

Molecular Genetics and Metabolism 106 (2012) 31–38

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

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

Induction of oxidative stress in brain of glutaryl-CoA dehydrogenase deficient mice by acute lysine administration

Bianca Seminotti [a], Mateus Struecker da Rosa [a], Carolina Gonçalves Fernandes [a], Alexandre Umpierrez Amaral [a], Luisa Macedo Braga [b], Guilhian Leipnitz [a], Diogo Onofre Gomes de Souza [a], Michael Woontner [c], David M. Koeller [d,e], Stephen Goodman [c], Moacir Wajner [a,f,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Fundação Estadual de Produção e Pesquisa em Saúde, Porto Alegre, RS, Brazil
[c] School of Medicine, University of Colorado Denver, Aurora, USA
[d] Department of Pediatrics, Oregon Health & Science University, Portland, OR, USA
[e] Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR, USA
[f] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 24 January 2012
Received in revised form 6 March 2012
Accepted 6 March 2012
Available online 13 March 2012

Keywords:
Glutaric acidemia type I
Glutaric acid
3-Hydroxyglutaric acid
Oxidative stress
Gcdh$^{-/-}$ mice

ABSTRACT

In the present work we evaluated a variety of indicators of oxidative stress in distinct brain regions (striatum, cerebral cortex and hippocampus), the liver, and heart of 30-day-old glutaryl-CoA dehydrogenase deficient (*Gcdh*$^{-/-}$) mice. The parameters evaluated included thiobarbituric acid-reactive substances (TBA-RS), 2-7-dihydrodichlorofluorescein (DCFH) oxidation, sulfhydryl content, and reduced glutathione (GSH) concentrations. We also measured the activities of the antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PD). Under basal conditions glutaric (GA) and 3-OH-glutaric (3OHGA) acids were elevated in all tissues of the *Gcdh*$^{-/-}$ mice, but were essentially absent in WT animals. In contrast there were no differences between WT and *Gcdh*$^{-/-}$ mice in any of the indicators or oxidative stress under basal conditions. Following a single intra-peritoneal (IP) injection of lysine (Lys) there was a moderate increase of brain GA concentration in *Gcdh*$^{-/-}$ mice, but no change in WT. Lys injection had no effect on brain 3OHGA in either WT or *Gcdh*$^{-/-}$ mice. The levels of GA and 3OHGA were approximately 40% higher in striatum compared to cerebral cortex in Lys-treated mice. In the striatum, Lys administration provoked a marked increase of lipid peroxidation, DCFH oxidation, SOD and GR activities, as well as significant reductions of GSH levels and GPx activity, with no alteration of sulfhydryl content, CAT and G6PD activities. There was also evidence of increased lipid peroxidation and SOD activity in the cerebral cortex, along with a decrease of GSH levels, but to a lesser extent than in the striatum. In the hippocampus only mild increases of SOD activity and DCFH oxidation were observed. In contrast, Lys injection had no effect on any of the parameters of oxidative stress in the liver or heart of *Gcdh*$^{-/-}$ or WT animals. These results indicate that in *Gcdh*$^{-/-}$ mice cerebral tissue, particularly the striatum, is at greater risk for oxidative stress than peripheral tissues following Lys administration.

1. Introduction

Glutaric aciduria type I (GA I, OMIM # 231670) is a cerebral organic aciduria caused by severe deficiency of glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) activity that catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA [1]. GCDH deficiency results in tissue accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3OHGA), and the derivative glutarylcarnitine. Lysine (Lys) oxidation seems to be quantitatively the most important pathway for the formation of these metabolites [2–4]. At birth, affected individuals present with macrocephaly associated with frontotemporal hypoplasia. Most untreated patients develop acute encephalopathic crises that lead to permanent striatal destruction and dystonia. The vast majority of these crises occur between 6 months and 3 years of age, and are frequently

Abbreviations: CAT, catalase; DCF-DA, 2-7-dihydrodichlorofluorescein diacetate; DCFH, 2-7-dihydrodichlorofluorescein; DTNB, 5-5-dithio-bis (2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; GA I, glutaric aciduria type I; GA, glutaric acid; *Gcdh*$^{-/-}$, deficient knockout mice; GCDH, glutaryl-CoA dehydrogenase; GPx, glutathione peroxidise; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; G6PD, glucose-6-phosphate dehydrogenase; 3OHGA, 3-hydroxyglutaric acid; IP, intra-peritoneal; KO, knockout; Lys, lysine; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NADP$^+$, nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffered saline; RS, reactive species; SPSS, Statistical Package for the Social Sciences; SOD, superoxide dismutase; TBA-RS, thiobarbituric acid-reactive substances; WT, wild type.

* Corresponding author at: 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. Fax: +55 51 3308 5535.
E-mail address: mwajner@ufrgs.br (M. Wajner).

1096-7192/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2012.03.002

precipitated by catabolic states, such as infections, fever and fasting. However, a number of patients who never have an acute crisis nonetheless develop dystonia due to striatal injury [5–7]. Another characteristic feature of GA I is delayed maturation of cerebral white matter, which also occurs in patients who never have an acute encephalopathic crisis. Pathologically the acute striatal degeneration in GA I is associated with loss of medium spiny neurons. Extra-striatal neuropathology commonly seen in GA I includes subdural and retinal hematomas, and a spongiform myelinopathy which seems to progresses with age [1,6,8–10].

Although the exact pathomechanisms underlying the brain damage of GA I are not fully understood, a great body of evidence indicates that GA and 3OHGA are involved in its pathogenesis, causing excitotoxicity, oxidative stress and mitochondrial dysfunction [4,11–33]. It is emphasized that most evidence revealing these pathomechanisms was based on in vitro experiments performed in fresh cerebral cortex and striatum, or in neuronal and astrocytic cell cultures from rats and chick embryos with normal GCDH activity, which makes the pathophysiological relevance of these works uncertain.

Recently a knockout (KO) model of GA I was developed in mice by replacing the *Gcdh* gene with an in-frame beta-galactosidase cassette [31]. Exposing these animals to high protein or Lys intake resulted in elevated serum and brain GA accumulation, as well as neuronal loss, myelin disruption and gliosis mostly in the striatum and deep cortex [33,34]. A chronic increase in oral Lys intake to weanling (4-week-old) *Gcdh*$^{-/-}$ mice provoked an increase of brain Lys and GA levels after 48 h of Lys exposure. Disrupted mitochondrial function, evidenced by mitochondrial swelling, accumulation of acetyl-coenzyme A, decrease of ATP, phosphocreatine and coenzyme A, as well as a reduction of alpha-ketoglutarate, glutamate, glutamine and GABA concentrations were also found in the cerebral cortex of the Lys-treated *Gcdh*$^{-/-}$ mice [34].

Considering that to the best of our knowledge practically nothing has been reported on cellular redox homeostasis in the *Gcdh*$^{-/-}$ genetic model, in the present study we evaluated a large spectrum of important parameters of oxidative stress in various brain regions (cerebral cortex, striatum and hippocampus) and in peripheral tissues (liver and heart) of WT and *Gcdh*$^{-/-}$ animals in order to clarify whether oxidative stress is involved in the pathogenesis, and more specifically in the brain damage of GA I. We measured thiobarbituric acid-reactive substances (TBA-RS), 2-7-dihydrodichlorofluorescein (DCFH) oxidation, sulfhydryl content, reduced glutathione (GSH) concentrations and the activities of the antioxidant enzymes glutathione peroxidise (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PD) in brain regions (cerebral cortex, striatum and hippocampus) and in peripheral tissues (liver and heart) of WT and *Gcdh*$^{-/-}$ mice while on standard mouse chow, and after acute lysine administration in order to clarify whether oxidative stress is involved in the pathogenesis and more specifically in the brain damage of GA I.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2–7.4 in the appropriate buffers for each technique.

2.2. Animals

Gcdh$^{-/-}$ and WT mice littermate controls, both of C129SvEv background, were generated from heterozygotes and maintained at Fundação Estadual de Produção e Pesquisa em Saúde (Porto Alegre, Brazil). 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 commercial chow containing 20% (w/w) protein, and 0.9% lysine (SUPRA, Porto Alegre, RS, Brazil). Thirty-day-old male WT and *Gcdh*$^{-/-}$ mice from F1 and F2 generations were used in all experiments.

2.3. Ethical statement

This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication no. 85-23, revised in 1996, and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Federal University of Rio Grande do Sul. All efforts were made to minimize suffering, discomfort, stress and the number of animals necessary to produce reliable scientific data.

2.4. Lys administration

A group of WT and *Gcdh*$^{-/-}$ animals were given one intraperitoneal (i.p.) injection of Lys (8 μmol/g) in order to investigate whether an acute Lys overload could induce oxidative stress in a model of GCDH deficiency. It is emphasized that catabolism leading to increased generation of Lys occurs during fasting/infections in GA I patients and may lead to striatal degeneration whose mechanisms are still poorly known. Lys, GA and 3OHGA concentrations were determined 1 h, 2 h and 24 h after Lys administration in striatum and cerebral cortex of WT and *Gcdh*$^{-/-}$ mice. Protein concentrations were also determined in brain homogenates from WT and *Gcdh*$^{-/-}$ animals. The obtained data revealed that these concentrations did not differ between controls and the GA I KO mice model and were approximately 3 mg/mL of protein. Lys concentrations were measured by cation-exchange chromatography on a Biochrom 30+ Amino Acid Analyzer, whereas GA and 3OHGA levels were quantified by stable isotope dilution GC/MS using an Agilent Technologies 6890N Gas Chromatograph equipped with a 5973N Mass Selective Detector. The internal standards were (2,2,4,4-D4) GA and (2,2,4,4-D4) 3OHGA. The parameters of oxidative stress were measured 24 h after Lys injection in order to study the medium to long-term effects of increased brain GA concentrations. It is important to emphasize that at this time GA concentrations returned to basal levels in the *Gcdh*$^{-/-}$ mice, so that our results could not be attributed to the presence of GA in the assays.

2.5. Tissue preparation

The mice were anesthetized with the mixture of ketamine (90 mg/kg) and xilazine (10 mg/kg) and intracardiacally perfused during 5 min with saline solution. After perfusion, brain, liver and heart were rapidly removed and placed on a Petri dish on ice. The olfactory bulb, pons, medulla, and cerebellum were discarded, and the cerebral cortex, striatum and hippocampus dissected and weighed. The tissues were homogenized in 9 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at $750 \times g$ for 10 min at 4 °C to discard nuclei and cell debris [35]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to measure oxidative stress parameters. Tissue slices (400 μm) were also prepared from the cerebral and peripheral structures for DCFH oxidation measurement.

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

TBA-RS levels were measured according to the method described by Yagi [36] with slight modifications. Briefly, 200 μL of 10% trichloroacetic acid and 300 μL of 0.67% TBA in 7.1% sodium sulfate were added to 100 μL of tissue supernatants containing 0.3 mg of protein and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex

was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 nm and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/mg protein. Results were expressed as percentage of controls.

2.7. 2-7-Dihydrodichlorofluorescein (DCFH) oxidation

Reactive species production was assessed according to LeBel et al. [37] by using 2-7-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA was prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl and incubated with tissue slices (30 mg) during 30 min at 37 °C. DCF-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2-7-dichlorofluorescein (DCF) in the presence of reactive species (RS). The DCF fluorescence intensity parallels to the amount of RS formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (0.2510 mM) and the levels of RS were calculated as pmol DCF formed/mg protein. Results were expressed as percentage of controls.

2.8. 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 [38]. Briefly, 30 μL of 10 mM DTNB and 980 μL of PBS were added to 50 μL of tissue supernatants containing 0.3 mg of protein. This was followed by 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were calculated and expressed as nmol/mg protein.

2.9. Reduced glutathione (GSH) levels

GSH levels were measured according to Browne and Armstrong [39]. Tissue supernatants with approximately 0.3 mg of protein were diluted (1:20, v/v) in 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation were incubated with an equal volume of o-phthaldialdehyde (1 mg/mL in methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol/mg protein. Results were expressed as percentage of controls.

2.10. Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel [40] 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/1 mM ethylenediaminetetraacetic acid, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH and tissue supernatants (approximately 3 μg of protein). One GPx unit (U) is defined as 1 μmol of NADPH consumed per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

2.11. Glutathione reductase (GR) activity

GR activity was measured according to Calberg and Mannervik [41] using oxidized glutathione (GSSG) and NADPH as substrates. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium with 200 mM sodium phosphate buffer, pH 7.5, containing 6.3 mM ethylenediaminetetraacetic acid, 1 mM GSSG, 0.1 mM NADPH and tissue supernatants (approximately 3 μg of protein). One GR unit (U) is defined as 1 μmol of GSSG reduced per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

2.12. Catalase (CAT) activity

CAT activity was assayed according to Aebi [42] 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 tissue supernatants (approximately 1 μg of protein). One unit (U) of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

2.13. Superoxide dismutase (SOD) activity

SOD activity was assayed according to Marklund [43] and is based on the capacity of pyrogallol to autooxidize, a process highly dependent on $O_2^{\bullet -}$, which is a substrate for SOD. The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase, 0.38 mM pyrogallol and tissue supernatants (approximately 1 μg of protein). A calibration curve was performed with purified SOD as standard to calculate the activity of SOD present in the samples. The results were calculated as U/mg protein and expressed as percentage of controls.

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

G6PD activity was measured by the method of Leong and Clark [44] in a reaction mixture (1 mL) containing 100 mM Tris–HCl pH 7.5, 10 mM MgCl2, 0.5 mM NADP + and tissue supernatants (approximately 3 μg of protein). The reaction was started by the addition of 1 mM glucose-6-phosphate and was followed in a spectrophotometer at 340 nm. One G6PD unit corresponds to 1 mmol of substrate transformed per minute and the specific activity was calculated as units per mg protein and expressed as percentage of controls.

2.15. Protein determination

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

2.16. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Data were analyzed using the Student's *t* test for unpaired samples. Only significant *t* 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.

3. Results

3.1. Effect of i.p. lysine injection on brain levels of lysine, glutaric acid and 3-hydroxyglutaric acid

In the cerebral cortex the levels of Lys were similar in WT and *Gcdh*$^{-/-}$ mice when maintained on standard lab chow, whereas GA and 3OHGA concentrations were undetectable in WT (results not

Table 1
Concentrations of lysine (Lys), glutaric acid (GA) and 3-hydroxyglutaric acid (3OHGA) in the cerebral cortex and striatum of WT and *Gcdh*$^{-/-}$ mice 2 h after a single intraperitoneal injection of lysine (8 μmol/g).

	Cerebral cortex			Striatum	
	WT plus Lys	*Gcdh*$^{-/-}$	*Gcdh*$^{-/-}$ plus Lys	WT plus Lys	*Gcdh*$^{-/-}$ plus Lys
Lys	48.5 ± 3.1	54 ± 1.8	85.2 ± 4.32** ##	63.5 ± 3.7	109 ± 5.76***
GA	248 ± 55.8	3709 ± 442	4974 ± 346*** ##	438 ± 63.4	6332 ± 355***
3OHGA	37 ± 6.72	340 ± 4.8	364 ± 98**	65.8 ± 23.2	491 ± 38.8***

Values are mean ± standard deviation for 4 independent experiments (animals). *P<0.05, **P<0.01, ***P<0.001, compared to WT plus Lys mice; ##P<0.01 compared to *Gcdh*$^{-/-}$ (Student's *t*-test for unpaired samples). We did not measure Lys, GA and 3OHGA in WT mice without Lys administration. Results are expressed as μmol/g brain.

shown). In contrast, cortical levels of GA and 3OHGA were strongly increased in the *Gcdh*$^{-/-}$ mice (15- and 9-fold, respectively), as has been previously observed in these animals [33,34]. Two hours after an acute i.p. injection of Lys the levels of Lys and GA in the cerebral cortex of *Gcdh*$^{-/-}$ animals were increased by an average 1.6 and 1.35 fold, respectively, with no significant change in the level of 3OHGA (Table 1). The levels of all three metabolites were similar at 1 and 2 h post Lys injection, and returned to baseline by 24 h (data not shown).

In the striatum, the levels of Lys, GA, and 3OHGA in *Gcdh*$^{-/-}$ mice were all significantly increased in comparison to WT mice 2 h after i.p. Lys injection. Striatal metabolite levels were also higher than those seen in the cerebral cortex of *Gcdh*$^{-/-}$ mice following i.p. Lys injection (Table 1). Data are not available for the cortical or striatal levels of these metabolites in WT mice that did not receive Lys supplementation and for the striatum of non-injected *Gcdh*$^{-/-}$ mice.

3.2. Effect of Lys injection on measures of oxidative stress

We first observed no differences between untreated WT and *Gcdh*$^{-/-}$ mice in any of the measured parameters at baseline (results not shown). We then evaluated the effect of a single i.p. injection of Lys on these parameters since this injection resulted in elevation of brain levels of GA and 3OHGA.

3.3. Lys administration induces brain lipid peroxidation in *Gcdh*$^{-/-}$ mice

In comparison to WT mice, the levels of malondialdehyde (TBA-RS) in *Gcdh*$^{-/-}$ mice were 50% higher in the cerebral cortex [$t_{(8)} = -3.952$; P<0.01] and 40% higher in the striatum [$t_{(12)} = -4.277$; P<0.001] following a single i.p. injection of Lys (Fig. 1). In contrast, TBA-RS levels

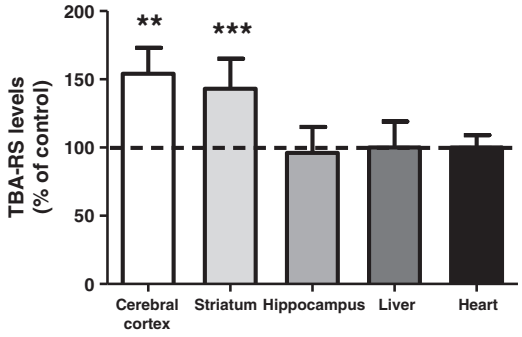


Fig. 1. Effect of lysine (Lys, 8 μmol/g) intraperitoneal administration on thiobarbituric acid-reactive substances (TBA-RS) levels in cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after injection. Values are mean ± standard deviation for 6 independent experiments (animals), expressed as percentage of control (Lys-injected *Gcdh*$^{+/+}$ mice) (dashed line) (Controls: 0.74 ± 0.18; [nmol TBA-RS/mg protein]). **P<0.01, ***P<0.001, compared to *Gcdh*$^{+/+}$ mice (Student's *t*-test for unpaired samples).

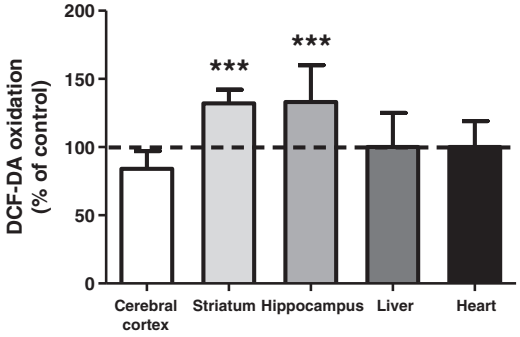


Fig. 2. Effect of lysine (Lys, 8 μmol/g) intraperitoneal administration on 2-7-dihydrodichlorofluorescein (DCFH) oxidation in cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after injection. Values are mean ± standard deviation for 6 independent experiments (animals), expressed as percentage of control (Lys-injected *Gcdh*$^{+/+}$ mice) (dashed line) (Controls: 20.99 ± 2.34; [pmol DCF/mg protein]). ***P<0.001, compared to *Gcdh*$^{+/+}$ mice (Student's *t*-test for unpaired samples).

in hippocampus, liver and heart from were the same in WT and *Gcdh*$^{-/-}$ mice 24 h after Lys injection.

3.4. Lys administration provokes brain DCFH oxidation in *Gcdh*$^{-/-}$ mice

Next, we assessed the influence of Lys injection on DCFH oxidation in cerebral cortex, striatum, hippocampus, liver and heart from *Gcdh*$^{-/-}$ mice. We observed that Lys significantly increased DCFH oxidation up to 30% in striatum [$t_{(12)} = -8.035$; P<0.001] and hippocampus [$t_{(9)} = -2.209$; P<0.001] of *Gcdh*$^{-/-}$ mice (Fig. 2). There were no differences between WT and *Gcdh*$^{-/-}$ mice in the amount of DCFH oxidation in cerebral cortex, liver and heart.

3.5. Lys administration does not alter sulfhydryl content in *Gcdh*$^{-/-}$ mice

We also investigated the effect of Lys administration on sulfhydryl content in striatum, cerebral cortex, hippocampus, liver and heart from *Gcdh*$^{-/-}$ and WT mice. It can be observed in Table 2 that Lys injection did not modify these parameters in all examined structures.

3.6. Lys administration reduces brain glutathione (GSH) concentrations in *Gcdh*$^{-/-}$ mice

We also investigated the effect of Lys administration on GSH concentrations in mice tissues. It can be observed in Fig. 3 that Lys injection significantly decreased (up to 15%) the concentrations of GSH in cerebral cortex [$t_{(10)} = 5.025$; P<0.001] and in striatum (up to 25%) [$t_{(8)} = 5.026$; P<0.001], with no alteration of this parameter in hippocampus, liver and heart.

Table 2
Sulfhydryl content of cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after intraperitoneal lysine injection (8 μmol/g).

	Sulfhydryl content	
	WT	*Gcdh*$^{-/-}$
Cerebral cortex	36.13 ± 6.92	36.76 ± 6.75
Striatum	59.54 ± 7.51	59.77 ± 12.61
Hippocampus	21.54 ± 9.39	24.52 ± 5.03
Liver	68.55 ± 4.98	72.89 ± 9.85
Heart	49.84 ± 6.19	50.28 ± 5.97

Values are mean ± standard deviation for 6 independent experiments (animals). Results are expressed as nmol/mg protein. No significant differences were found between groups (Student's *t*-test for unpaired samples).

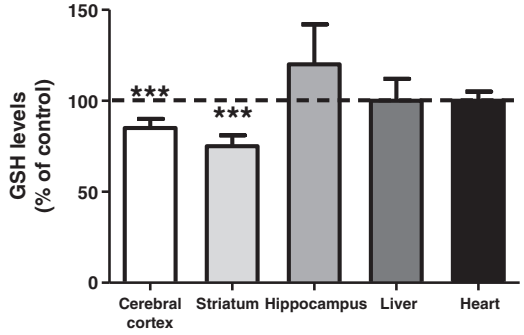


Fig. 3. Effect of lysine (Lys, 8 μmol/g) intraperitoneal administration on reduced glutathione (GSH) levels in cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after injection. Values are mean ± standard deviation for 6 independent experiments (animals), expressed as percentage of control (Lys-injected *Gcdh*$^{+/+}$ mice) (dashed line) (Controls: 7.62 ± 0.45; [nmol GSH/mg protein]). ***$P<0.001$, compared to *Gcdh*$^{+/+}$ mice (Student's *t*-test for unpaired samples).

3.7. Lys administration alters the activities of the antioxidant enzymes glutathione peroxidase, glutathione reductase and superoxide dismutase in brain of *Gcdh*$^{-/-}$ mice

We investigated the effect of Lys administration on the activities of GPx, GR, CAT, SOD and G6PD in cerebral cortex, striatum, hippocampus, liver and heart from *Gcdh*$^{-/-}$ mice. Our results demonstrate that a single Lys injection provoked an increase of SOD activity in cerebral cortex (28%) [$t_{(7)} = -3.192$; $P<0.05$], striatum (100%) [$t_{(12)} = -5.922$; $P<0.001$] and hippocampus (22%) [$t_{(8)} = 2.613$; $P<0.05$] (Fig. 4D), with no alteration in liver and heart (results not shown). Moreover, the activity of GPx was significantly decreased (41%) [$t_{(12)} = 6.947$; $P<0.001$] (Fig. 4A) and GR activity was increased (45%) only in the striatum after Lys administration ([$t_{(12)} = -4.708$; $P<0.001$] (Fig. 4B), with no alterations in cerebral cortex, hippocampus, liver and heart (results not shown). Finally, CAT (Fig. 4C) and G6PD (Fig. 4E) activities were not altered by Lys administration in any of the tissues examined.

4. Discussion

In the present work we searched for alterations of cellular redox homeostasis in central and peripheral tissues of 30-day-old *Gcdh*$^{-/-}$ mice. We first observed that the oxidative stress parameters did not differ between *Gcdh*$^{-/-}$ and WT mice receiving a normal diet (0.9% Lys). These data reflect that at this age under basal conditions there is no induction of oxidative stress in the *Gcdh*$^{-/-}$ mice compared to the WT mice.

We also investigated the effects of an acute Lys injection on oxidative damage, reactive oxygen species formation and the antioxidant defenses in the *Gcdh*$^{-/-}$ mice since temporary high Lys generation due to enhanced proteolysis occurs in GA I patients during episodes of metabolic decompensation, which are generally followed by acute striatum degeneration. Lys administration led to increased brain levels of GA soon after injection, with the highest concentrations achieved in the striatum from the *Gcdh*$^{-/-}$ mice. We also found that Lys administration resulted in increased reactive species production, lipid peroxidation and impaired antioxidant defenses following Lys supplementation in the brain of the *Gcdh*$^{-/-}$ animals. Furthermore, the observed effects were more pronounced in the striatum, but were also seen in the cerebral cortex and in hippocampus to a much lesser degree, with no alteration in liver and heart in, as compared to the WT mice.

Our observations that Lys overload provoked marked lipid peroxidation in the striatum and cerebral cortex of *Gcdh*$^{-/-}$ mice was based on the increased TBA-RS levels that reflect a high formation of malondialdehyde, an end product of membrane fatty acid oxidation [46]. It is feasible that the lipid oxidative damage following Lys supplementation could be secondary to elevated amounts of reactive species since DCFH-DA oxidation, a measurement of these species, was found increased especially in the in the striatum of the Lys-injected *Gcdh*$^{-/-}$ animals.

Regarding to the antioxidant defenses, the concentrations of GSH, the major brain antioxidant with both cytoplasmatic and mitochondrial GSH pools [47], were reduced in the striatum and cerebral cortex of the *Gcdh*$^{-/-}$ animals that received Lys. Considering that GSH is an

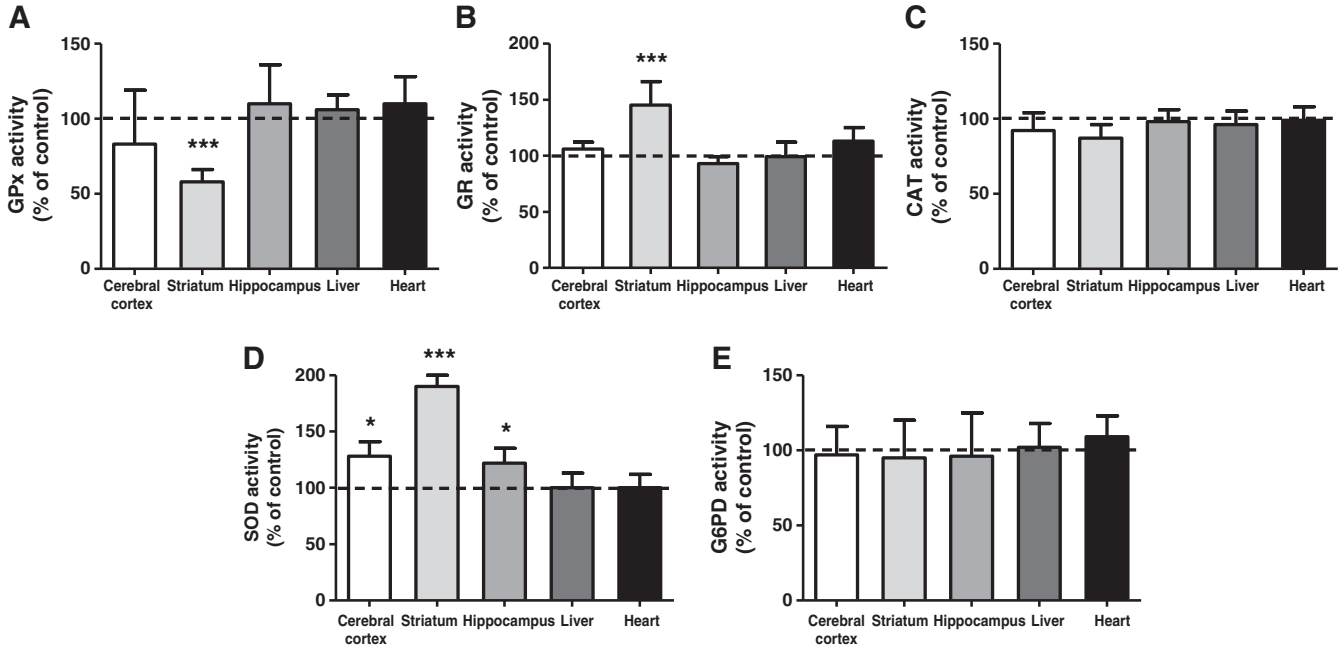


Fig. 4. Effect lysine (Lys, 8 μmol/g) intraperitoneal administration on the activity of the antioxidant enzymes glutathione peroxidase (GPx; A), glutathione reductase (GR; B), catalase (CAT; C), superoxide dismutase (SOD; D) and glucose-6-phosphate dehydrogenase (G6PD; E) in cerebral cortex, striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice 24 h after injection. Values are mean ± standard deviation for 6 independent experiments (animals), expressed as percentage of control (Lys-injected *Gcdh*$^{+/+}$ mice) (dashed line) (Controls: [A: 4.28 ± 1.41]; [B: 20.10 ± 0.63]; [C: 4.93 ± 0.59]; [D: 9.91 ± 1.21]; [E: 6.59 ± 1.15]; [U/mg protein]). ***$P<0.001$, compared to *Gcdh*$^{+/+}$ mice (Student's *t*-test for unpaired samples).

effective scavenger of free radicals, a protector of thiol groups and a cofactor of GPx, α-tocopherol and melatonin [48,49], it is concluded that Lys overload reduced the brain tissue antioxidant defenses in the *Gcdh*$^{-/-}$ mice model. Furthermore, since a disruption of GSH system homeostasis may result in oxidative injury in sensitized neurons [50,51], it is presumed that our findings of reduced GSH levels in striatum and cerebral cortex caused by Lys administration in *Gcdh*$^{-/-}$ mice may be at least in part involved in the brain abnormalities observed in Lys-treated *Gcdh*$^{-/-}$ mice [4,32,33,52].

Furthermore, GPx activity was decreased and GR activity increased in the striatum, whereas SOD activity was augmented in all brain structures evaluated of the Lys-treated *Gcdh*$^{-/-}$ animals. We presume that induction of the expression of GR and SOD at the gene level might have taken place as a compensatory mechanism in response to increased formation of reactive species. This hypothesis is base on the observations that reactive oxygen species have previously been shown to regulate the expression of numerous genes, including those encoding antioxidant enzymes, via specific signaling mechanisms [47,53,54].

It must be considered that the brain has low activity of antioxidant enzymes and reduced content of non-enzymatic antioxidants [47], implying a lower capacity to react against free radicals compared with other tissues. The brain has also a high rate of oxidative metabolism coupled to ROS production, high amount of iron and greater peroxidation potential because of its elevated content of polyunsaturated fatty acids [46,55] that makes this tissue more vulnerable to increased formation of reactive species [55].

Since oxidative stress is caused by the imbalance between pro-oxidants and anti-oxidant mechanisms [46], our present data strongly indicate that acute Lys surplus induces in vivo oxidative stress in the striatum and cerebral cortex of *Gcdh*$^{-/-}$ mice. It must be emphasized here that reactive oxygen species are capable to oxidize different molecules, including lipids, proteins and DNA, representing a key event in the pathogenic cascade leading to apoptotic (under chronic oxidative stress) or necrotic (under acute oxidative insults) cell death [56–58].

Interestingly, oxidative stress accompanies inflammatory processes [59–61] and patients with GA I frequently develop striatal damage during and after infections that are associated with inflammation and with a potential increase of tissue concentrations of GA and 3OHGA [18,62,63]. Furthermore, astroglyosis, a characteristic feature of neuroinflammation, is a common histopathological finding in the *Gcdh*$^{-/-}$ mice following exposure to chronically increased dietary Lys intake [33]. It has been also demonstrated that oxidative stress impairs the intrinsic cell potential, leading to proinflammatory and proapoptotic signals and creating a vicious circle between oxidative stress and neuroinflammation [64–66].

Our results showing that *Gcdh*$^{-/-}$ animals receiving acute Lys surplus resulted in 40% increase of GA in the brain, but not elevation of 3OHGA, suggest that probably GA induced oxidative damage and reduced the antioxidant defenses in these animals. It is emphasized that Lys easily crosses the blood brain barrier and is oxidized to GA in neural cells [18]. This is in agreement with the findings of Zinnanti and colleagues [34] demonstrating that high dietary Lys intake induces increased brain levels of GA which temporally parallels the mitochondrial abnormalities observed in striatum and cerebral cortex of 4-week-old *Gcdh*$^{-/-}$ mice. The significant body of in vitro and in vivo evidence showing that GA and 3OHGA provoke lipid and protein oxidation and reduce the antioxidant defenses in brain of 30-day-old rats reinforces the view that the accumulating metabolites in GA I, particularly GA, are mainly responsible for the brain damage in this disorder [21,27–29].

An intriguing issue in our results was why under basal levels no difference was encountered in the oxidative stress parameters between KO and WT mice. We feel that the inability to document any differences does not rule out the possibility that the chronic exposure of the *Gcdh*$^{-/-}$ mice to elevated GA and 3OHGA does lead to compensatory changes that we were unable to demonstrate, and that such compensatory mechanisms reduce their vulnerability to the chronic toxicity of the accumulated metabolites. Nonetheless, it is apparent that an acute Lys injection does provoke oxidative stress in the *Gcdh*$^{-/-}$ animals, suggesting that the rapid increase in GA (20-fold higher than in WT mice) overwhelms the antioxidant capacity of the brains of these mice.

Our observation that oxidative damage in the brains of *Gcdh*$^{-/-}$ mice was dependent on an increase in brain lysine and GA concentrations suggests that therapies aimed at reducing brain lysine uptake could be effective for treatment of GA I. Interestingly, it has been previously shown that arginine, which competes with lysine for brain transport, and glucose that also decreased brain lysine uptake, possibly by reduced utilization and compartmentalization, have beneficial effects in the mouse GA I model and in human patients [33,34,67,68].

5. Conclusions

In conclusion, the present results provide for the first time experimental evidence that oxidative stress is induced in vivo in the brain, and particularly in the striatum, of *Gcdh*$^{-/-}$ mice after acute Lys supplementation. It is therefore presumed that overproduction of reactive radicals is potentially deleterious to the CNS and may represent a relevant pathomechanism of brain injury in GA I and particularly during encephalopathic crises triggered by infections which are accompanied by high brain increases of GA concentrations. Thus, trials with antioxidants, especially those targeted to the mitochondria [69,70], considered the main source of reactive oxygen species, could be undertaken initially in the *Gcdh*$^{-/-}$ mice model and may potentially represent a novel adjuvant therapy together with Lys dietary restriction and L-carnitine supplementation in the treatment of GA I patients, in order to protect children from the devastating clinical sequelae of striatal damage.

Conflicts of interest

There are no conflicts of interest.

Acknowledgments

We are grateful to the financial support of CNPq, PROPESq/UFRGS, FAPERGS, PRONEX, FINEP Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00, and Instituto Nacional de Ciência e Tecnologia-Excitotoxicidade e Neuroproteção (INCT-EN).

References

[1] S.I. Goodman, M.D. Norenberg, R.H. Shikes, D.J. Breslich, P.G. Moe, Glutaric aciduria: biochemical and morphologic considerations, J. Pediatr. 90 (1977) 746–750.
[2] K.P. Blemings, T.D. Crenshaw, N.J. Benevenga, Mitochondrial lysine uptake limits hepatic lysine oxidation in rats fed diets containing 5, 20 or 60% casein, J. Nutr. 128 (1998) 2427–2434.
[3] K.A. Strauss, Glutaric aciduria type 1: a clinician's view of progress, Brain 128 (2005) 697–699.
[4] P. Jafari, O. Braissant, L. Bonafé, D. Ballhausen, The unsolved puzzle of neuropathogenesis in glutaric aciduria type I, Mol. Genet. Metab. 104 (2011) 425–437.
[5] K.A. Strauss, J. Lazovic, M. Wintermark, D.H. Morton, Multimodal imaging of striatal degeneration in Amish patients with glutaryl-CoA dehydrogenase deficiency, Brain 130 (2007) 1905–1920.
[6] I. Harting, E. Neumaier-Probst, A. Seitz, E.M. Maier, B. Assmann, I. Baric, M. Troncoso, C. Mühlhausen, J. Zschocke, N.P. Boy, G.F. Hoffmann, S.F. Garbade, S. Kölker, Dynamic changes of striatal and extrastriatal abnormalities in glutaric aciduria type I, Brain 132 (2009) 1764–1782.
[7] J. Heringer, S.P. Boy, R. Ensenauer, Use of guidelines improves the neurological outcome in glutaric aciduria type I, Ann. Neurol. 68 (2010) 743–752.
[8] S. Kimura, M. Hara, A. Nezu, H. Osaka, S. Yamazaki, K. Saitoh, Two cases of glutaric aciduria type 1: clinical and neuropathological findings, J. Neurol. Sci. 123 (1994) 38–43.
[9] S. Kölker, G.F. Hoffmann, D.S. Schor, P. Feyh, L. Wagner, I. Jeffrey, M. Pourfarzam, J.G. Okun, J. Zschocke, I. Baric, M.D. Bain, C. Jakobs, R.A. Chalmers, Glutaryl-CoA dehydrogenase deficiency: region-specific analysis of organic acids and acylcarnitines in post mortem brain predicts vulnerability of the putamen, Neuropediatrics 34 (2003) 253–260.

[10] D.M. Koeller, M. Woontner, L.S. Crnic, B. Kleinschmidt-DeMasters, J. Stephens, E.L. Hunt, S.I. Goodman, Biochemical, pathologic and behavioral analysis of a mouse model of glutaric acidemia type I, Hum. Mol. Genet. 11 (2002) 347–357.
[11] O. Stokke, S.I. Goodman, P.G. Moe, Inhibition of brain glutamate decarboxylase by glutarate, glutaconate, and beta-hydroxyglutarate: explanation of the symptoms in glutaric aciduria? Clin. Chim. Acta 66 (1976) 411–415.
[12] S. Kölker, B. Ahlemeyer, J. Krieglstein, G.F. Hoffmann, 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors in vitro, J. Inherit. Metab. Dis. 22 (1999) 259–262.
[13] S. Kölker, B. Ahlemeyer, J. Krieglstein, G.F. Hoffmann, Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons, Pediatr. Res. 50 (2001) 76–82.
[14] S. Kölker, B. Ahlemeyer, R. Hühne, E. Mayatepek, J. Krieglstein, G.F. Hoffmann, Potentiation of 3-hydroxyglutarate neurotoxicity following induction of astrocytic iNOS in neonatal rat hippocampal cultures, Eur. J. Neurosci. 13 (2001) 2115–2122.
[15] S. Kölker, J.G. Okun, B. Ahlemeyer, A.T. Wyse, F. Hörster, M. Wajner, D. Kohlmüller, E. Mayatepek, J. Krieglstein, G.F. Hoffmann, Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons, J. Neurosci. Res. 68 (2002) 424–431.
[16] S. Kölker, G. Köhr, B. Ahlemeyer, J.G. Okun, V. Pawlak, F. Hörster, E. Mayatepek, J. Krieglstein, G.F. Hoffmann, Ca^{2+} and Na^{+} dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons, Pediatr. Res. 52 (2002) 199–206.
[17] S. Kölker, D.M. Koeller, S. Sauer, F. Hörster, M.A. Schwab, G.F. Hoffmann, K. Ullrich, J.G. Okun, Excitotoxicity and bioenergetics in glutaryl-CoA dehydrogenase deficiency, J. Inherit. Metab. Dis. 27 (2004) 805–812.
[18] S. Kölker, S.W. Sauer, J.G. Okun, G.F. Hoffmann, D.M. Koeller, Lysine intake and neurotoxicity in glutaric aciduria type I: towards a rationale for therapy? Brain 129 (2006) e54.
[19] L.O. Porciúncula, A. Dal-Pizzol Jr., A.S. Coitinho, T. Emanuelli, D.O. Souza, M. Wajner, Inhibition of synaptosomal [^{3}H]glutamate uptake and [^{3}H]glutamate binding to plasma membranes from brain of young rats by glutaric acid in vitro, J. Neurol. Sci. 173 (2000) 93–96.
[20] L.O. Porciúncula, T. Emanuelli, R.G. Tavares, C. Schwarzbold, M.E. Frizzo, D.O. Souza, M. Wajner, Glutaric acid stimulates glutamate binding and astrocytic uptake and inhibits vesicular glutamate uptake in forebrain from young rats, Neurochem. Int. 45 (2004) 1075–1086.
[21] F. de Oliveira Marques, M.E. Hagen, C.D. Pederzolli, A.M. Sgaravatti, K. Durigon, C.G. Testa, C.M. Wannmacher, A.T. de Souza Wyse, M. Wajner, C.S. Dutra-Filho, Glutaric acid induces oxidative stress in brain of young rats, Brain Res. 964 (2003) 153–158.
[22] R.B. Rosa, C. Schwarzbold, K.B. Dalcin, G.C. Ghisleni, C.A. Ribeiro, M.B. Moretto, M.E. Frizzo, G.F. Hoffmann, D.O. Souza, M. Wajner, Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats, Neurochem. Int. 45 (2004) 1087–1094.
[23] R.B. Rosa, K.B. Dalcin, A.L. Schmidt, D. Gerhardt, C.A. Ribeiro, G.C. Ferreira, P.F. Schuck, A.T. Wyse, L.O. Porciúncula, S. Wofchuk, C.G. Salbego, D.O. Souza, M. Wajner, Evidence that glutaric acid reduces glutamate uptake by cerebral cortex of infant rats, Life Sci. 81 (2007) 1668–1676.
[24] M. Wajner, S. Kölker, D.O. Souza, G.F. Hoffmann, C.F. de Mello, Modulation of glutamatergic and GABAergic neurotransmission in glutaryl-CoA dehydrogenase deficiency, J. Inherit. Metab. Dis. 27 (2004) 825–828.
[25] K.B. Dalcin, R.B. Rosa, A.L. Schmidt, J.S. Winter, G. Leipnitz, C.S. Dutra-Filho, C.M. Wannmacher, L.O. Porciúncula, D.O. Souza, M. Wajner, Age and brain structural related effects of glutaric and 3-hydroxyglutaric acids on glutamate binding to plasma membranes during rat brain development, Cell. Mol. Neurobiol. 27 (2007) 805–818.
[26] M.R. Fighera, L.F. Royes, A.F. Furian, M.S. Oliveira, N.G. Fiorenza, R. Frussa-Filho, J.C. Petry, R.C. Coelho, C.F. Mello, GM1 ganglioside prevents seizures, Na^{+}, K^{+}-ATPase activity inhibition and oxidative stress induced by glutaric acid and pentylenetetrazole, Neurobiol. Dis. 22 (2006) 611–623.
[27] A. Latini, R. Borba Rosa, K. Scussiato, S. Llesuy, A. Belló-Klein, M. Wajner, 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats, Brain Res. 956 (2002) 367–373.
[28] A. Latini, K. Scussiato, G. Leipnitz, C.S. Dutra-Filho, M. Wajner, Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum, J. Inherit. Metab. Dis. 28 (2005) 57–67.
[29] A. Latini, G.C. Ferreira, K. Scussiato, P.F. Schuck, A.F. Solano, C.S. Dutra-Filho, C.R. Vargas, M. Wajner, Induction of oxidative stress by chronic and acute glutaric acid administration to rats, Cell. Mol. Neurobiol. 27 (2007) 423–438.
[30] S. Olivera, A. Fernandez, A. Latini, J.C. Rosillo, G. Casanova, M. Wajner, P. Cassina, L. Barbeito, Astrocytic proliferation and mitochondrial dysfunction induced by accumulated glutaric acidemia I (GAI) metabolites: possible implications for GAI pathogenesis, Neurobiol. Dis. 32 (2008) 528–534.
[31] D.V. Magni, A.F. Furian, M.S. Oliveira, M.A. Souza, F. Lunardi, J. Ferreira, C.F. Mello, L.F. Royes, M.R. Fighera, Kinetic characterization of l-[(3)H]glutamate uptake inhibition and increase oxidative damage induced by glutaric acid in striatal synaptosomes of rats, Int. J. Dev. Neurosci. 27 (2009) 65–72.
[32] S. Olivera-Bravo, A. Fernández, M.N. Sarlabós, J.C. Rosillo, G. Casanova, M. Jiménez, L. Barbeito, Neonatal astrocyte damage is sufficient to trigger progressive striatal degeneration in a rat model of glutaric acidemia-I, PLoS One 6 (2011) e20831.
[33] W.J. Zinnanti, J. Lazovic, E.B. Wolpert, D.A. Antonetti, M.B. Smith, J.R. Connor, M. Woontner, S.I. Goodman, K.C. Cheng, A diet-induced mouse model for glutaric aciduria type I, Brain 129 (2006) 899–910.
[34] W.J. Zinnanti, J. Lazovic, C. Housman, Mechanism of age-dependent susceptibility and novel treatment strategy in glutaric acidemia type I, J. Clin. Invest. 117 (2007) 3258–3270.
[35] P. Evelson, M. Travacio, M. Repetto, J. Escobar, S. Llesuy, E.A. Lissi, Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols, Arch. Biochem. Biophys. 388 (2001) 261–266.
[36] K. Yagi, Simple procedure for specific assay of lipid hydroperoxides in serum or plasma, Methods Mol. Biol. 108 (1998) 107–110.
[37] C.P. LeBel, H. Ischiropoulos, S.C. Bondy, Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress, Chem. Res. Toxicol. 5 (1992) 227–231.
[38] M.Y. Aksenov, W.R. Markesbery, Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease, Neurosci. Lett. 302 (2001) 141–145.
[39] R.W. Browne, D. Armstrong, Reduced glutathione and glutathione disulfide, Methods Mol. Biol. 108 (1998) 347–352.
[40] A. Wendel, Glutathione peroxidase, Methods Enzymol. 77 (1981) 325–332.
[41] I. Calberg, B. Mannervik, Glutathione reductase, Methods Enzymol. 113 (1985) 484–490.
[42] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.
[43] S.L. Marklund, Pyrogallol autoxidation, Handbook for Oxygen Radical Research, CRC Press, Boca Raton, FL, 1985, pp. 243–247.
[44] S.F. Leong, J.B. Clark, Regional development of glutamate dehydrogenase in the rat brain, J. Neurochem. 43 (1984) 106–111.
[45] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[46] B. Halliwell, J.M.C. Gutteridge, Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Oxford University Press Inc., Oxford, 2007, pp. 187–340.
[47] B. Halliwell, Oxidative stress and neurodegeneration: where are we now? J. Neurochem. 97 (2006) 1634–1658.
[48] R. Dringen, L. Kussmaul, J.M. Gutterer, J. Hirrlinger, B. Hamprecht, The glutathione system of peroxide detoxification is less efficient in neurons than in astroglial cells, J. Neurochem. 72 (1999) 2523–2530.
[49] A.W. Siu, R.J. Reiter, C.H. To, The efficacy of vitamin E and melatonin as antioxidants against lipid peroxidation in rat retinal homogenates, J. Pineal Res. 24 (1998) 239–244.
[50] J.B. de Haan, C. Bladier, P. Griffiths, M. Kelner, R.D. O'Shea, N.S. Cheung, R.T. Bronson, M.J. Silvestro, S. Wild, S.S. Zheng, P.M. Beart, P.J. Hertzog, I. Kola, Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide, J. Biol. Chem. 273 (1998) 22528–22536.
[51] P.J. Crack, K. Cimdins, U. Ali, P.J. Hertzog, R.C. Iannello, Lack of glutathione peroxidase-1 exacerbates Abeta-mediated neurotoxicity in cortical neurons, J. Neural Transm. 113 (2006) 645–657.
[52] W.J. Zinnanti, J. Lazovic, Mouse model of encephalopathy and novel treatment strategies with substrate competition in glutaric aciduria type I, Mol. Genet. Metab. (Suppl. 1) (2010) S88–S91.
[53] T.H. Rushmore, M.R. Morton, C.B. Pickett, The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity, J. Biol. Chem. 266 (1991) 11632–11639.
[54] V. Lakshminarayanan, E.A. Drab-Weiss, K.A. Roebuck, H_2O_2 and tumor necrosis factor-alpha induce differential binding of the redox-responsive transcription factors AP-1 and NF-kappaB to the interleukin-8 promoter in endothelial and epithelial cells, J. Biol. Chem. 273 (1998) 32670–32678.
[55] B. Halliwell, Reactive oxygen species and the central nervous system, J. Neurochem. 59 (1992) 1609–1623.
[56] J.T. Coyle, P. Puttfarcken, Oxidative stress, glutamate, and neurodegenerative disorders, Science 262 (1993) 689–695.
[57] M.F. Beal, Energetics in the pathogenesis of neurodegenerative diseases, Trends Neurosci. 23 (2000) 298–304.
[58] M.V. Frantseva, J.L. Perez Velazquez, G. Tsoraklidis, A.J. Mendonca, Y. Adamchik, L.R. Mills, P.L. Carlen, M.W. Burnham, Oxidative stress is involved in seizure-induced neurodegeneration in the kindling model of epilepsy, Neuroscience 97 (2000) 431–435.
[59] K.L. Double, S. Reyes, E.L. Werry, G.M. Halliday, Selective cell death in neurodegeneration: why are some neurons spared in vulnerable regions? Prog. Neurobiol. 92 (2010) 316–329.
[60] A.A. Farooqui, Studies on plasmalogen-selective phospholipase A2 in brain, Mol. Neurobiol. 41 (2010) 267–273.
[61] T. Farooqui, A.A. Farooqui, Lipid-mediated oxidative stress and inflammation in the pathogenesis of Parkinson's disease, Park. Dis. (2011) 247467 ID.
[62] K.A. Strauss, E.G. Puffenberger, D.L. Robinson, D.H. Morton, Type I glutaric aciduria, part 1: natural history of 77 patients, Am. J. Med. Genet. C Semin. Med. Genet. 121C (2003) 38–52.
[63] S.W. Sauer, J.G. Okun, G. Fricker, A. Mahringer, I. Müller, L.R. Crnic, C. Mühlhausen, G.F. Hoffmann, F. Hörster, S.I. Goodman, C.O. Harding, D.M. Koeller, S. Kölker, 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 (2006) 899–910.
[64] S. Rosales-Corral, R.J. Reiter, D.X. Tan, G.G. Ortiz, G. Lopez-Armas, Functional aspects of redox control during neuroinflammation, Antioxid. Redox Signal. 13 (2010) 193–247.
[65] P. Bermejo, S. Martin-Aragon, J. Benedi, C. Susin, E. Felici, P. Gil, J.M. Ribera, A.M. Villar, Peripheral levels of glutathione and protein oxidation as markers in the development of Alzheimer's disease from mild cognitive impairment, Free Radic. Res. 42 (2008) 162–170.

[66] T. Satoh, S.A. Lipton, Redox regulation of neuronal survival mediated by electrophilic compounds, Trends Neurosci. 30 (2007) 37–45.
[67] S.W. Sauer, S. Opp, G.F. Hoffmann, D.M. Koeller, J.G. Okun, S. Kölker, Therapeutic modulation of cerebral L-lysine metabolism in a mouse model for glutaric aciduria type I, Brain 134 (2011) 157–170.
[68] K.A. Strauss, J. Brumbaugh, A. Duffy, B. Wardley, D. Robinson, C. Hendrickson, S. Tortorelli, A.B. Moser, E.G. Puffenberger, N.L. Rider, D.H. Morton, Safety, efficacy and physiological actions of a lysine-free, arginine-rich formula to treat glutaryl-CoA dehydrogenase deficiency: focus on cerebral amino acid influx, Mol. Genet. Metab. 104 (2011) 93–106.
[69] C. Cortés-Rojo, A.R. Rodríguez-Orozco, Importance of oxidative damage on the electron transport chain for the rational use of mitochondria-targeted antioxidants, Mini Rev. Med. Chem. 11 (2011) 625–632.
[70] K. Min, A.J. Smuder, O.S. Kwon, A.N. Kavazis, H.H. Szeto, S.K. Powers, Mitochondrial-targeted antioxidants protect the skeletal muscle against immobilization-induced muscle atrophy, J. Appl. Physiol. 111 (2011) 1459–1466.

Capítulo III

Disruption of brain redox homeostasis in glutaryl-CoA dehydrogenase deficient mice treated with high dietary lysine supplementation

Bianca Seminotti, Alexandre Umpierrez Amaral, Mateus Struecker da Rosa, Carolina Gonçalves Fernandes, Guilhian Leipnitz, Silvia Olivera-Bravo, Luis Barbeito, César Augusto Joao Ribeiro, Diogo Onofre Gomes de Souza, Michael Woontner, David M Koeller, Stephen Goodman, Moacir Wajner.

Artigo científico publicado no periódico

Molecular Genetics and Metabolism

Molecular Genetics and Metabolism 108 (2013) 30–39

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

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

Disruption of brain redox homeostasis in glutaryl-CoA dehydrogenase deficient mice treated with high dietary lysine supplementation

Bianca Seminotti [a], Alexandre Umpierrez Amaral [a], Mateus Struecker da Rosa [a], Carolina Gonçalves Fernandes [a], Guilhian Leipnitz [a], Silvia Olivera-Bravo [b], Luis Barbeito [b], César Augusto J. Ribeiro [a], Diogo Onofre Gomes de Souza [a], Michael Woontner [c], Stephen I. Goodman [c], David M. Koeller [d], Moacir Wajner [a,e,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay
[c] School Medicine University of Colorado Denver, Aurora, USA
[d] Departments of Pediatrics, Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR, USA
[e] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 13 September 2012
Received in revised form 1 November 2012
Accepted 1 November 2012
Available online 9 November 2012

Keywords:
Glutaric acidemia type I
Glutaric acid
High lysine diet
Oxidative stress
Gcdh$^{-/-}$ mice

ABSTRACT

Deficiency of glutaryl-CoA dehydrogenase (GCDH) activity or glutaric aciduria type I (GA I) is an inherited neurometabolic disorder biochemically characterized by predominant accumulation of glutaric acid and 3-hydroxyglutaric acid in the brain and other tissues. Affected patients usually present acute striatum necrosis during encephalopathic crises triggered by metabolic stress situations, as well as chronic leukodystrophy and delayed myelination. Considering that the mechanisms underlying the brain injury in this disease are not yet fully established, in the present study we investigated important parameters of oxidative stress in the brain (cerebral cortex, striatum and hippocampus), liver and heart of 30-day-old GCDH deficient knockout (*Gcdh*$^{-/-}$) and wild type (WT) mice submitted to a normal lysine (Lys) (0.9% Lys), or high Lys diets (2.8% or 4.7% Lys) for 60 h. It was observed that the dietary supplementation of 2.8% and 4.7% Lys elicited noticeable oxidative stress, as verified by an increase of malondialdehyde concentrations (lipid oxidative damage) and 2-7-dihydrodichlorofluorescein (DCFH) oxidation (free radical production), as well as a decrease of reduced glutathione levels and alteration of various antioxidant enzyme activities (antioxidant defenses) in the cerebral cortex and the striatum, but not in the hippocampus, the liver and the heart of *Gcdh*$^{-/-}$ mice, as compared to WT mice receiving the same diets. Furthermore, alterations of oxidative stress parameters in the cerebral cortex and striatum were more accentuated in symptomatic, as compared to asymptomatic *Gcdh*$^{-/-}$ mice exposed to 4.7% Lys overload. Histopathological studies performed in the cerebral cortex and striatum of these animals exposed to high dietary Lys revealed increased expression of oxidative stress markers despite the absence of significant structural damage. The results indicate that a disruption of redox homeostasis in the cerebral cortex and striatum of young *Gcdh*$^{-/-}$ mice exposed to increased Lys diet may possibly represent an important pathomechanism of brain injury in GA I patients under metabolic stress.

1. Introduction

Glutaric aciduria type I (GA I, McKusick 23167; OMIM #231670) is a neurometabolic disorder caused by a severe deficiency of the activity of glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) due to heterogeneous mutations in the GCDH gene. It is biochemically characterized by tissue accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3OHGA) and glutarylcarnitine and high urinary excretion of GA and 3OHGA. Some GA I patients only excrete 3OHGA in the urine. Affected patients present macrocephaly associated with frontotemporal atrophy at birth. Between 6 months and 3 years most untreated children suffer acute encephalopathic crises commonly precipitated by infectious illness or vaccination. These episodes coincide with massive striatal necrosis, clinically manifested

Abbreviations: CAT, catalase; DCF-DA, 2-7-dihydrodichlorofluorescein diacetate; DCFH, 2-7-dihydrodichlorofluorescein; DTNB, 5-5-dithio-bis (2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; GA I, glutaric aciduria type I; GA, glutaric acid; *Gcdh*$^{-/-}$, deficient knockout mice; GCDH, glutaryl-CoA dehydrogenase; GFAP, glial fibrillary acidic protein; GPx, glutathione peroxidise; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; G6PD, glucose-6-phosphate dehydrogenase; 3OHGA, 3-hydroxyglutaric acid; KO, knockout; Lys, lysine; NADPH, nicotinamide adenine dinucleotide phosphate reduced; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NeuN, neuronal nuclei; PAF, paraformaldehyde; PBS, phosphate buffered saline; PBST, PBS plus 0.1–0.3% Triton X-100; SPSS, Statistical Package for the Social Sciences; SOD, superoxide dismutase; TBA-RS, thiobarbituric acid-reactive substances; WT, wild type.

* Corresponding author at: 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. Fax: +55 51 3308 5535.
E-mail address: mwajner@ufrgs.br (M. Wajner).

1096-7192/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymgme.2012.11.001

as dystonia and dyskinesia and other neurological symptoms [1–3]. Patients can also develop chronically progressive neurological dysfunction without undergoing acute episodes [2,4,5]. Cranial MRI findings usually show a pattern of progressive spongiform white matter changes (leukoencephalopathy) with cortical hypoplasia, subdural hemorrhages and degeneration of the basal ganglia with loss of medium spiny neurons, as well as astrogliosis [4,6–9].

Despite a great deal of experimental work on GA I neuropathology in the last few years, the comprehension of the pathomechanisms of brain injury in this disorder still remains partial. However, it has been suggested that the accumulating organic acids GA and 3OHGA behave as neurotoxins through three major synergistic mechanisms, excitotoxicity, disruption of mitochondrial energy homeostasis and oxidative stress in vulnerable regions of the brain [10–35]. However, the pathophysiological significance of these data is uncertain since most data were obtained in tissues with normal GCDH activity, whereas some results are contradictory [22,36] possibly due to differences between the experimental approaches utilized.

A knockout (KO) mice model of GA I ($Gcdh^{-/-}$) developed by Koeller and collaborators [37] proved to mimic the biochemical phenotype (accumulation of GA and 3OHGA in tissues and body fluids) and some of the neurological abnormalities (leukoencephalopathy) observed in GA I patients, but did not induce the characteristic striatum necrosis even under metabolic or infectious stressors. Zinnanti and colleagues [38,39] improved this model by exposing the KO animals to high protein or lysine (Lys) (4.7%) dietary intake that gave rise to higher brain GA concentrations and striatum degeneration. It was also shown that the neuropathological findings observed in the high dietary Lys-treated mice were comparable to those found in human patients, including neuronal loss and vacuolization, blood–brain barrier breakdown, myelin disruption and gliosis mostly in the striatum and deep cortex. These investigators also demonstrated a disturbance of mitochondrial energy metabolism, revealed by lower concentrations of ATP and phosphocreatine and alterations of other bioenergetics markers in the KO mice supplemented by high Lys [39]. However, a setback of this model is that most 4-week-old $Gcdh^{-/-}$ mice receiving high Lys supplementation presented hypoactivity, followed by paralysis, seizures and death after 5–7 days, paralleled with striatal and cortical pathological abnormalities after 48 h of this diet. These clinical and pathological findings may possibly interfere with the investigation of the underlying mechanisms of brain damage in this model since the obtained results may be the consequence rather than the cause of neural cell injury and death.

We recently found that the striatum and to a lesser degree the cerebral cortex of 30-day-old $Gcdh^{-/-}$ mice are vulnerable to oxidative damage 24 h after an acute Lys intraperitoneal administration (8 μmol/g) [40]. It is of note that some of these animals were symptomatic, presenting hypoactivity. In the present study we aimed to evaluate a large spectrum of important parameters of redox homeostasis in brain structures (cerebral cortex, striatum and hippocampus) and in peripheral tissues (liver and heart) from 30-day-old $Gcdh^{-/-}$ and WT mice submitted to a normal (0.9%) or high (2.8 or 4.7%) Lys diets for 60 h. We used a moderately increased amount of Lys dietary supplementation (2.8% Lys), besides the proposed high diet with 4.7% Lys supplementation, because the later diet usually gives rise to severe neurological alterations and provokes death in the $Gcdh^{-/-}$ mice [38,39]. Histopathological studies were also carried out in the brain of these animals submitted to a high (4.7%) Lys diet.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2–7.4 in the appropriate buffers used for each technique.

2.2. Animals

$Gcdh^{-/-}$ and WT mice of C129SvEv background were generated from heterozygotes and maintained at Unidade Experimental Animal of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). The animals were kept 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% protein (w/w) commercial chow. At 27 days of age, WT and $Gcdh^{-/-}$ animals were submitted to a normal (0.9% Lys) or high dietary Lys intake (2.8 or 4.7% Lys) for 60 h. WT and $Gcdh^{-/-}$ mice were killed 60 h after the beginning of the diet and then the parameters of oxidative stress were immediately measured in the various tissues (see detailed protocol below).

2.3. Ethical statement

This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of the United States of America, NIH, publication n 85-23, revised in 2011, and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Hospital de Clínicas de Porto Alegre. All efforts were made to minimize suffering, discomfort, stress and the number of animals necessary to produce reliable scientific data.

2.4. Tissue preparation

The mice were anesthetized with the mixture of ketamine (90 mg/kg) and xilazine (10 mg/kg) and intracardially perfused during 5 min with saline solution. After perfusion, the brain, liver and heart were rapidly removed and placed on a Petri dish on ice. The olfactory bulb, pons, medulla, and cerebellum were discarded from the total brain, and the cortex, striatum and hippocampus dissected and weighed. The tissues were homogenized in 9 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at $750 \times g$ for 10 min at 4 °C to discard nuclei and cell debris [41]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to measure oxidative stress parameters. We emphasize that supernatants are widely used as a valid model system to evaluate important pro-oxidant and antioxidant parameters of oxidative stress [41–46] because they contain the whole cell machinery including preserved organelles such as mitochondria (the major source of free radical generation) and enzymes that are necessary for free radical production and scavenging [43,46–48]. Tissue slices (400 μm) were also prepared from the cerebral cortex and the peripheral structures for DCFH oxidation measurement.

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

TBA-RS levels were measured according to the method described by Yagi [49] with slight modifications. Briefly, 200 μL of 10% trichloroacetic acid and 300 μL of 0.67% TBA in 7.1% sodium sulfate were added to 100 μL of tissue supernatants containing 0.3 mg of protein and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 nm and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/ mg protein. Results were expressed as percentage of controls.

2.6. 2-7-Dihydrodichlorofluorescein (DCFH) oxidation

Reactive species production was assessed according to LeBel et al. [50] by using 2-7-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA was prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl and incubated with tissue slices (30 mg) during 30 min at 37 °C. DCF-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2-7-dichlorofluorescein (DCF) in the presence of reactive species. The DCF fluorescence intensity parallels to the amount of reactive species formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (0.25–10 mM) and the levels of reactive species were calculated as pmol DCF formed/mg protein. Results were expressed as percentage of controls.

2.7. Reduced glutathione (GSH) levels

GSH levels were measured according to Browne and Armstrong [51]. Tissue supernatants with approximately 0.3 mg of protein were diluted (1:20, v/v) in 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 for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol/ mg protein. Results were expressed as percentage of controls.

2.8. Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel [52] 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 containing 1 mM ethylenediaminetetraacetic acid, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH and tissue supernatants (approximately 3 μg of protein). One GPx unit (U) is defined as 1 μmol of NADPH consumed per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

2.9. Superoxide dismutase (SOD) activity

SOD activity was assayed according to Marklund [53] and is based on the capacity of pyrogallol to autooxidize, a process highly dependent on $O_2^{\bullet-}$, which is a substrate for SOD. The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer containing 1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase, 0.38 mM pyrogallol and tissue supernatants (approximately 1 μg of protein). A calibration curve was performed with purified SOD as standard to calculate the activity of SOD present in the samples. The results were calculated as U/mg protein and expressed as percentage of controls.

2.10. Catalase (CAT) activity

CAT activity was assayed according to Aebi [54] 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 tissue supernatants (approximately 1 μg of protein). One unit (U) of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute. The specific activity was calculated as U/mg protein. Results were expressed as percentage of controls.

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

G6PD activity was measured by the method of Leong and Clark [55] in a reaction mixture containing 100 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$ and tissue supernatants (approximately 3 μg of protein). The reaction was started by the addition of 1 mM glucose-6-phosphate and was followed in a spectrophotometer at 340 nm. One G6PD unit corresponds to 1 mmol of substrate transformed per minute and the specific activity was calculated as U/mg protein and expressed as percentage of controls.

2.12. Histopathological studies

WT and *Gcdh*$^{-/-}$ mice were anesthetized with 90:10 mg/kg ketamine/xilazine mixture and intracardially perfused with 4% paraformaldehyde (PAF) in 0.01 M PBS, pH 7.4. After fixation, brains were quickly removed, maintained overnight at 4 °C in 10% PAF and changed to phosphate buffered saline (PBS) until sectioning. A 1000S Leica vibratome was used to obtain 30–50 μm thick consecutive coronal series. Sections were stored either free-floating at 4 °C or mounted on gelatin-coated slides for histological analysis. Representative sections were stained with 1% boraxic methylene blue to evidence gross neuroanatomical features. In some experiments, the cerebral cortex and striatum were dissected. Myelin content was estimated by histochemistry against Sudan III and Sudan IV (Sudan Black). Immunohistochemistry was performed in striatum to study the different cell populations and oxidative stress prototypic markers. For each animal and staining procedure, 5 to 8 equivalent sections were immunostained. Striatal neuronal population was recognized by labeling against the pan-neuronal marker neuronal nuclei (NeuN), whereas the astrocytic population was studied by using antibodies against S100β and glial fibrillary acidic protein (GFAP). Microglia was recognized by Iba1 immunoreactivity. Immunostaining against Ki67 was performed to look for proliferating cells. A monoclonal antibody reported as recognizing GCDH positive cells were also employed. To perform immunohistochemical assays, free-floating sections were washed with PBS, submitted to antigen retrieval by boiling in 10 mM pH 6.0 sodium citrate during 10 min, then washed twice for 10 min. After that, slices were permeabilized with PBS plus 0.1–0.3% Triton X-100 (PBST) and treated with blocking buffer (PBS + 0.3% Triton X-100 + 5% bovine serum albumin) for 30 min. Finally slices were incubated with pairs of antibodies i.e. anti-NeuN (Millipore, 1:250) together with anti-GFAP (1:400), or anti-Ki67 (1:200, Abcam), or anti-S100β (1:500) with anti-nitrotyrosine (Millipore, 1:300), or anti-Ki67 (Abcam, 1:300) with anti-iNOS (Santa Cruz, 1:300). All dilutions were made in PBST. After a 4 °C overnight incubation, sections were rinsed in PBS, and incubated at room temperature for 90 min with 1:800 dilutions of corresponding secondary antibodies conjugated to fluorescent probes (Molecular Probes). Sections were then washed, mounted in glycerol and imaged in a FV300 Olympus confocal microscope provided with 405, 488, 546 and 633 nm lasers. Primary or secondary antibodies were omitted in negative controls [29].

2.13. Protein determination

Protein concentrations were measured by the method of Lowry et al. [56] using bovine serum albumin as standard.

2.14. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Data were analyzed using the Student's *t* test for unpaired samples to compare the parameters between and WT and *Gcdh*$^{-/-}$ mice. Only significant *t* 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.

3. Results

We first found that WT mice treated with 0.9%, 2.8% or 4.7% dietary Lys did not present apparent motor or behavioral alterations. The same occurred with the *Gcdh*$^{-/-}$ mice receiving normal or 2.8% Lys intake. In contrast, 20–25% of *Gcdh*$^{-/-}$ mice fed a 4.7% Lys became hypoactive approximately 48–60 h after the diet start. Unless otherwise stated, the results presented below refer to asymptomatic mice. We also observed in a separate experiment that the hypoactive mice died after approximately 5–7 days of diet.

Regarding the oxidative stress parameters, we observed that these biochemical measurements did not differ in all examined structures (cerebral cortex, striatum, hippocampus, liver and heart) from asymptomatic WT and *Gcdh*$^{-/-}$ mice receiving normal chow (0.9% Lys). However, significant alterations of most of these parameters were observed in the brain of asymptomatic *Gcdh*$^{-/-}$ mice receiving 2.8% or 4.7% Lys for 60 h, as discussed below with more details. Furthermore, there were no differences in the magnitude of the alterations of the oxidative stress parameters in *Gcdh*$^{-/-}$ mice fed with either 2.8% or 4.7% Lys, implying that a "moderately" increased Lys intake (2.8%) was sufficient to impair redox homeostasis in the absence of symptoms.

3.1. Lys dietary overload induces lipid peroxidation in the cerebral cortex and striatum of *Gcdh*$^{-/-}$ mice

Lys overload (2.8% and 4.7%) resulted in a significant increase of malondialdehyde (TBA-RS) levels in the cerebral cortex [2.8%: $t_{(8)}=3.313$; $P<0.01$; 4.7%: $t_{(8)}=5.049$; $P<0.001$] (Fig. 1A) and in striatum [4.7%: $t_{(8)}=3.501$; $P<0.01$] (Fig. 1B) of *Gcdh*$^{-/-}$ mice as compared to WT mice. In contrast, this measurement was not altered in the hippocampus, liver and heart of *Gcdh*$^{-/-}$ animals receiving 2.8% or 4.7% Lys diet (results not shown).

3.2. Lys dietary overload increases reactive species formation (DCFH oxidation) in the cerebral cortex of *Gcdh*$^{-/-}$ mice

We also observed that DCFH oxidation was significantly increased in the cerebral cortex of *Gcdh*$^{-/-}$ mice receiving 2.8% or 4.7% Lys dietary supplementation, as compared to WT [2.8%: $t_{(8)}=4.646$; $P<0.01$; 4.7%: $t_{(8)}=-2.412$; $P<0.05$] (Fig. 2). However, this parameter was not changed in liver and heart of *Gcdh*$^{-/-}$ mice receiving 2.8% or 4.7% Lys dietary supplementation (results not shown).

3.3. Lys dietary overload decreases reduced glutathione (GSH) levels in the cerebral cortex and striatum of *Gcdh*$^{-/-}$ mice

We verified that GSH levels were markedly diminished in the cerebral cortex [2.8%: $t_{(8)}=2.626$; $P<0.05$; 4.7%: $t_{(8)}=3.390$; $P<0.01$] and striatum [2.8%: $t_{(8)}=3.555$; $P<0.05$; 4.7%: $t_{(8)}=2.764$; $P<0.01$] of *Gcdh*$^{-/-}$ that received 2.8% or 4.7% Lys dietary intake as compared to WT mice (Figs. 3A and B). In contrast, GSH values were not changed in the hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice treated with 2.8% or 4.7% Lys diet (results not shown).

3.4. GSH levels and DCFH oxidation are inversely correlated in the cerebral cortex of *Gcdh*$^{-/-}$ mice on a high Lys diet

Furthermore, the reduction of GSH concentrations (decrease of the most important brain antioxidant defense) was inversely correlated with DCFH oxidation (increased production of reactive species) ($r=-0.6662$, $P<0.05$), suggesting that free radicals were probably involved in the decrease of the non-enzymatic antioxidant defenses (Fig. 4).

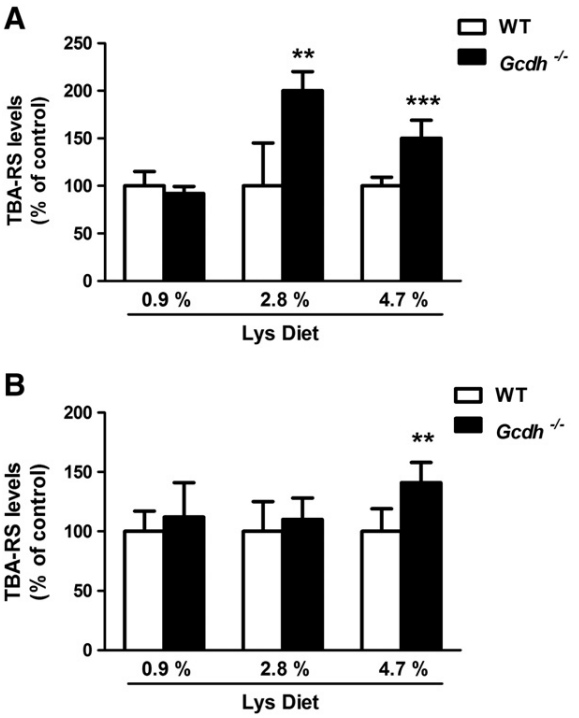


Fig. 1. Effect of lysine (Lys) dietary overload on thiobarbituric acid-reactive substances (TBA-RS) levels in the cerebral cortex (A) and the striatum (B) of WT and *Gcdh*$^{-/-}$ mice. Mice were fed with 0.9% (normal), 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet. Values are mean ± standard deviation for 5 independent experiments (animals) expressed as percentage of control (Control: A 0.9%: 1.49 ± 0.28; 2.8%: 1.12 ± 0.11; 4.7%: 1.30 ± 0.10; B 0.9%: 1.25 ± 0.21; 2.8%: 0.89 ± 0.22; 4.7%: 1.12 ± 0.21; [nmol TBA-RS/mg protein]). $^{**}P<0.01$, $^{***}P<0.001$, compared to WT mice (Student's *t*-test for unpaired samples).

3.5. Lys dietary overload increases the activities of superoxide dismutase and catalase in the cerebral cortex of *Gcdh*$^{-/-}$ mice

High Lys intake (2.8% and 4.7%) induced a significant increase in the activities of SOD and CAT in the cerebral cortex of *Gcdh*$^{-/-}$ mice [SOD [2.8% Lys: $t_{(8)}=2.835$; $P<0.05$; 4.7% Lys: $t_{(8)}=2.719$; $P<0.05$]; CAT [2.8% Lys: $t_{(8)}=4.253$; $P<0.01$; 4.7% Lys: [$t_{(12)}=3.424$; $P<0.01$]] (Figs. 5B and C). In contrast, no alterations of these antioxidant enzyme activities were found in striatum, hippocampus, liver and heart of *Gcdh*$^{-/-}$ mice receiving either 2.8% or 4.7% Lys dietary supplementation (results not shown). Furthermore, GPx and G6PD activities were not changed in the cerebral cortex (Figs. 5A and D),

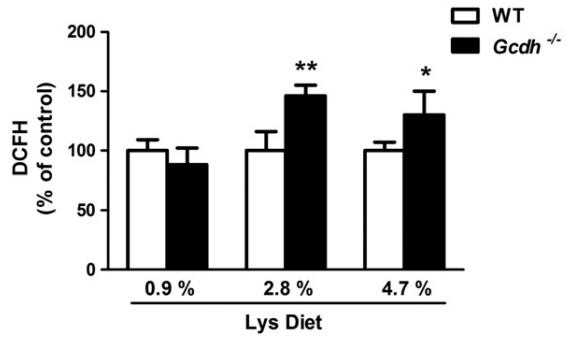


Fig. 2. Effect of lysine (Lys) dietary overload on 2-7-dihydrodichlorofluorescein (DCFH) oxidation in the cerebral cortex of WT and *Gcdh*$^{-/-}$ mice. Mice were fed with 0.9% (normal), 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet. Values are mean ± standard deviation for 5 independent experiments (animals), expressed as percentage of control (Control: 0.9%: 8.64 ± 0.89; 2.8%: 6.59 ± 0.87; 4.7%: 7.12 ± 0.52; [pmol DCF/mg protein]). $^{*}P<0.05$, $^{**}P<0.01$, compared to WT mice (Student's *t*-test for unpaired samples).

as well as in the striatum, hippocampus and in the peripheral tissues (heart and liver) of *Gcdh*$^{-/-}$ mice receiving 0.9%, 2.8% or 4.7% Lys, as compared to WT mice (results not shown).

3.6. Disruption of redox homeostasis is significantly greater in the cerebral cortex and striatum of symptomatic Gcdh$^{-/-}$ *mice submitted to 4.7% Lys dietary overload*

In a separate set of experiments we submitted *Gcdh*$^{-/-}$ mice submitted to a 4.7% Lys diet and compared the changes of redox homeostasis between symptomatic and asymptomatic animals. Fig. 6 shows that alterations of oxidative stress parameters were more evident in the cerebral cortex and striatum of symptomatic as compared to asymptomatic *Gcdh*$^{-/-}$ mice submitted to 4.7% Lys dietary overload for 60 h. We can observe in the figure a significantly higher TBA-RS ($t_{(9)} = 7.174$, $P<0.001$) and SOD activity ($t_{(9)} = 6.505$, $P<0.001$) and a nonsignificant tendency of GSH reduction in symptomatic *Gcdh*$^{-/-}$ mice in the cerebral cortex. The striatum of symptomatic *Gcdh*$^{-/-}$ mice presented a significant reduction of GSH levels ($t_{(9)} = 4.658$, $P<0.001$), an increase of SOD activity ($t_{(9)} = 3.391$, $P<0.01$) and a nonsignificant increase of TBA-RS levels as compared to asymptomatic *Gcdh*$^{-/-}$ mice.

3.7. Lys dietary overload does not affect gross brain anatomy and white matter integrity in the cerebral cortex and striatum but provokes oxidative damage in Gcdh$^{-/-}$ *mice submitted to 4.7% Lys dietary overload*

We found no significant changes neither in the gross brain anatomy of both WT and *Gcdh*$^{-/-}$ mice submitted to high Lys nor in the myelination in the cerebral cortex and striatum of high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice, and no ventricular enlargement, indicating that this short treatment (60 h) did not significantly affect the gross white matter integrity of these brain structures (data not shown). Furthermore, the number of striatal neurons (estimated by immunostaining against the panneuronal marker NeuN per area unit) remained unchanged in high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice as compared to WT. However, NeuN neuronal staining in high Lys treated animals was weaker and occupied cell cytoplasm. A significant injury response was also discarded since Ki67 immunostaining was similar in WT and *Gcdh*$^{-/-}$ mice (Fig. 7A). Furthermore, no changes in S100b+ and GFAP+ cell number were observed in high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice, discarding prominent astrogliosis, despite that S100+ cells appeared swollen with increased immunoreactivity (Fig. 7B). Interestingly, the striatum of high Lys (4.7%) exposed *Gcdh*$^{-/-}$ mice showed an increased number of neurons stained with nitrotyrosine (Fig. 8A), as well as stained with iNOS, indicating an induction of oxidative damage (Fig. 8B). Furthermore, *Gcdh*$^{-/-}$ positive neurons were observed only in WT mice, as expected (Fig. 8B, left panel).

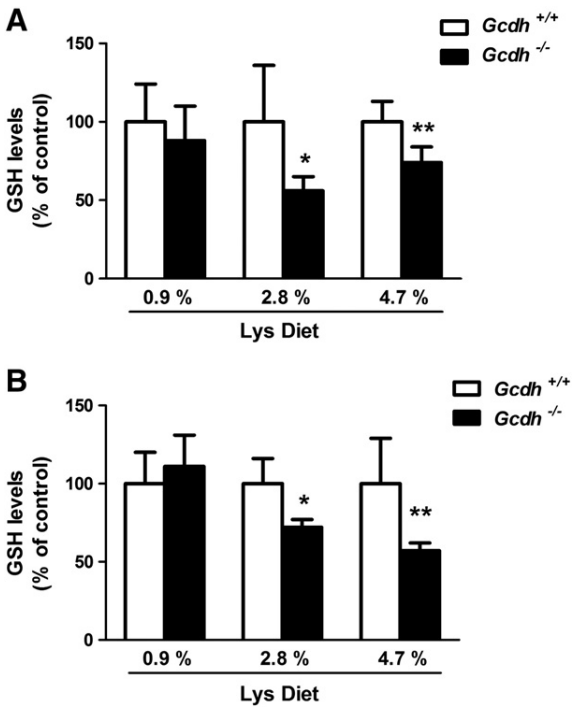


Fig. 3. Effect of lysine (Lys) dietary overload on GSH levels in the cerebral cortex (A) and the striatum (B) of WT and *Gcdh*$^{-/-}$ mice. Mice were fed with 0.9% (normal), 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet. Values are mean ± standard deviation for 5 independent experiments (animals), expressed as percentage of control (Control: A 0.9%: 8.35 ± 2.56; 2.8%: 9.74 ± 3.57; 4.7%: 5.86 ± 0.79; B 0.9%: 10.2 ± 0.95; 2.8%: 16.2 ± 2.71; 4.7%: 12.8 ± 2.93; [nmol GSH/mg protein]). *$P<0.05$, **$P<0.01$, compared to WT mice (Student's *t*-test for unpaired samples).

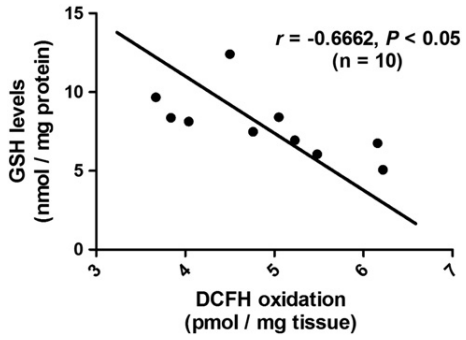


Fig. 4. Correlation between GSH levels and DCFH oxidation in the cerebral cortex of *Gcdh*$^{-/-}$ *mice on a high Lys diet*. Mice were fed with a 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet and GSH concentrations (nmol/mg protein) and DCFH oxidation (pmol/mg tissue) correlated.

4. Discussion

A large number of works investigated the molecular mechanisms implicated in the neuropathology of GA I, but the exact underlying processes are still poorly established probably because most studies were carried out in the brain tissue with normal GCDH activity.

Considering that astrogliosis (reactive astrocytes), that is usually associated with increased production of reactive oxygen species, was found in the striatum and cerebral cortex of the *Gcdh*$^{-/-}$ mice submitted to 4.7% dietary Lys [39] and also in the brain of GA I patients [4,8,9], it seems justified to investigate redox homeostasis in Lys-treated *Gcdh*$^{-/-}$ mice. In this regard, we have recently verified that oxidative stress is induced in vivo in the striatum and cerebral cortex of 30-day-old *Gcdh*$^{-/-}$ mice submitted to an acute intraperitoneal injection of Lys [40]. However, some of these results were obtained in tissues from symptomatic mice with hypoactivity and partial paralysis. In the present work we comprehensively evaluated oxidative stress parameters in brain structures (cerebral cortex, striatum and hippocampus) and peripheral tissues (liver and heart) from young (30-old-day) asymptomatic WT and *Gcdh*$^{-/-}$ mice chronically exposed to a normal (0.9% Lys) or special diets enriched with Lys (2.8% and 4.7%) for 60 h.

The utilization of a moderately increased (2.8%) Lys diet aimed to test whether this treatment could induce disruption of redox homeostasis without causing severe symptoms or death in the Lys supplemented animals, which occurs in *Gcdh*$^{-/-}$ mice receiving a 4.7% Lys enriched dietary supplementation [38]. It is of note that 2.8% Lys-treated animals did not present symptoms, whereas the 4.7% Lys dietary supplementation provoked hypoactivity in approximately one fourth of the *Gcdh*$^{-/-}$ mice at the time of sacrifice (60 h of diet). However, our objective was first to compare parameters of redox homeostasis between WT and *Gcdh*$^{-/-}$ asymptomatic mice, and therefore we only used animals with no symptoms in these experiments.

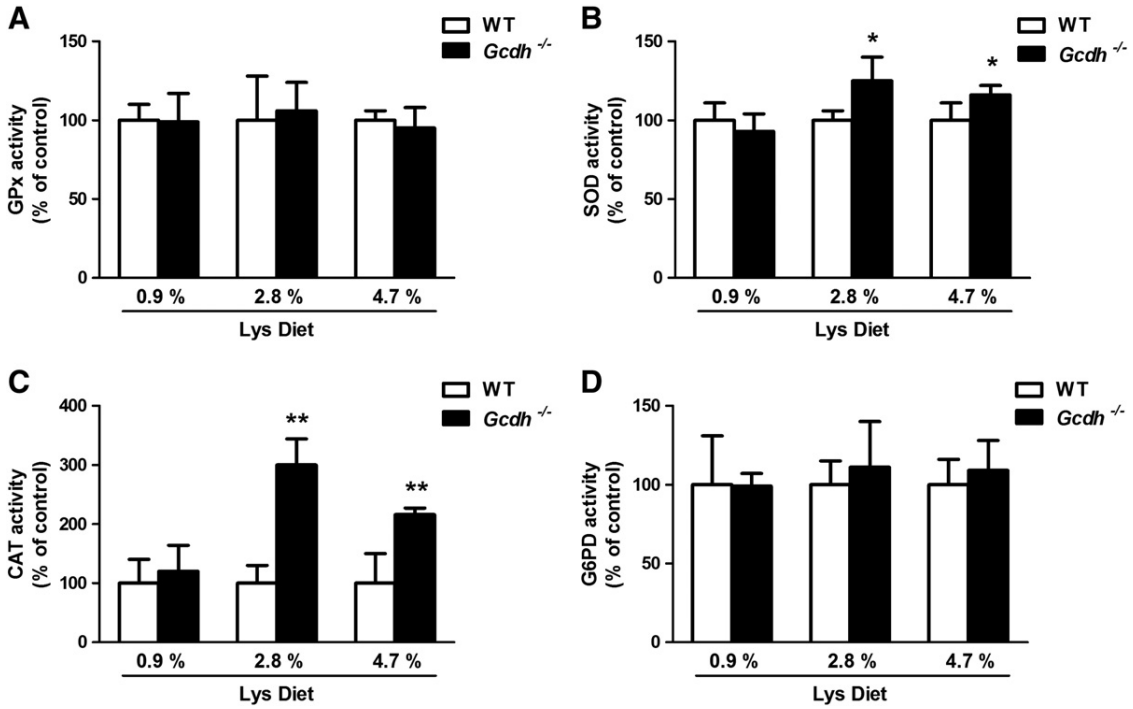


Fig. 5. Effects of lysine (Lys) dietary overload on the activity of the antioxidant enzymes glutathione peroxidase (GPx; A), superoxide dismutase (SOD; B), catalase (CAT; C) and glucose-6-phosphate dehydrogenase (G6PD; D) in the cerebral cortex of WT and *Gcdh*$^{-/-}$ mice. Mice were fed with 0.9% (normal), 2.8% (3-fold increased) or 4.7% (5-fold increased) Lys diet. Values are mean ± standard deviation for 5 independent experiments (animals), expressed as percentage of control (Control: GPx: 0.9%: 14.3 ± 1.46; 2.8%: 12.0 ± 3.36; 4.7%: 13.7 ± 0.90; SOD: 0.9%: 7.90 ± 0.86; 2.8%: 6.83 ± 0.43; 4.7%: 6.98 ± 0.78; CAT: 0.9%: 0.46 ± 0.03; 2.8%: 0.30 ± 0.12; 4.7%: 0.73 ± 0.18; G6PD: 0.9%: 5.34 ± 1.00; 2.8%: 4.76 ± 0.72; 4.7%: 4.93 ± 0.82; [U/mg protein]). *P<0.05, **P<0.01, compared to WT mice (Student's t-test for unpaired samples).

Overall we found that the evaluated parameters of redox homeostasis did not differ between WT and *Gcdh*$^{-/-}$ mice receiving a normal chow (0.9% Lys) in all brain structures and peripheral tissues, which is in accordance with our previous results [40] and reinforces the view that under basal conditions cellular redox status is preserved in the *Gcdh*$^{-/-}$ animals under a normal diet. However, chronic dietary Lys overload provoked a selective disruption of redox homeostasis in the brain. We observed that asymptomatic *Gcdh*$^{-/-}$ mice exposed to either a 2.8% or a 4.7% Lys enriched diet presented significant and similar alterations of oxidative stress parameters in the cerebral cortex and striatum, but not in hippocampus, liver and heart, as compared to WT mice. The data described in details below indicate that 2.8% Lys diet was sufficient to compromise redox homeostasis in *Gcdh*$^{-/-}$ mice and that the brain is more vulnerable to Lys overload, as compared to peripheral structures.

Significantly increased TBA-RS (malondialdehyde) levels were demonstrated in the striatum and cerebral cortex of *Gcdh*$^{-/-}$ mice after 60 h of high (2.8% or 4.7%) Lys diet. These findings reflect

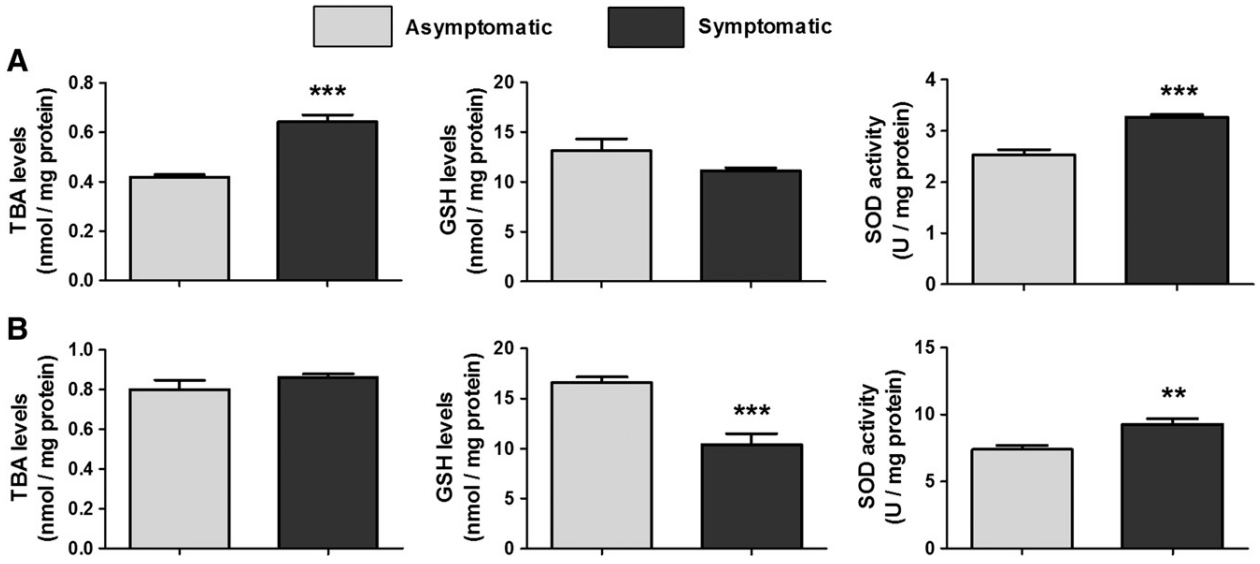


Fig. 6. Oxidative stress parameters (TBA-RS levels, GSH concentrations and SOD activity) in the cerebral cortex (A) and the striatum (B) of asymptomatic and symptomatic *Gcdh*$^{-/-}$ mice fed a 4.7% Lys diet. Values are mean ± standard deviation for 5–6 independent experiments (animals). Data were presented as nmol TBA/mg protein, nmol GSH/mg protein and U/mg protein for TBA-RS levels, GSH concentrations and SOD activity, respectively. **P<0.01, ***P<0.001, compared to asymptomatic mice (Student's t-test for unpaired samples).

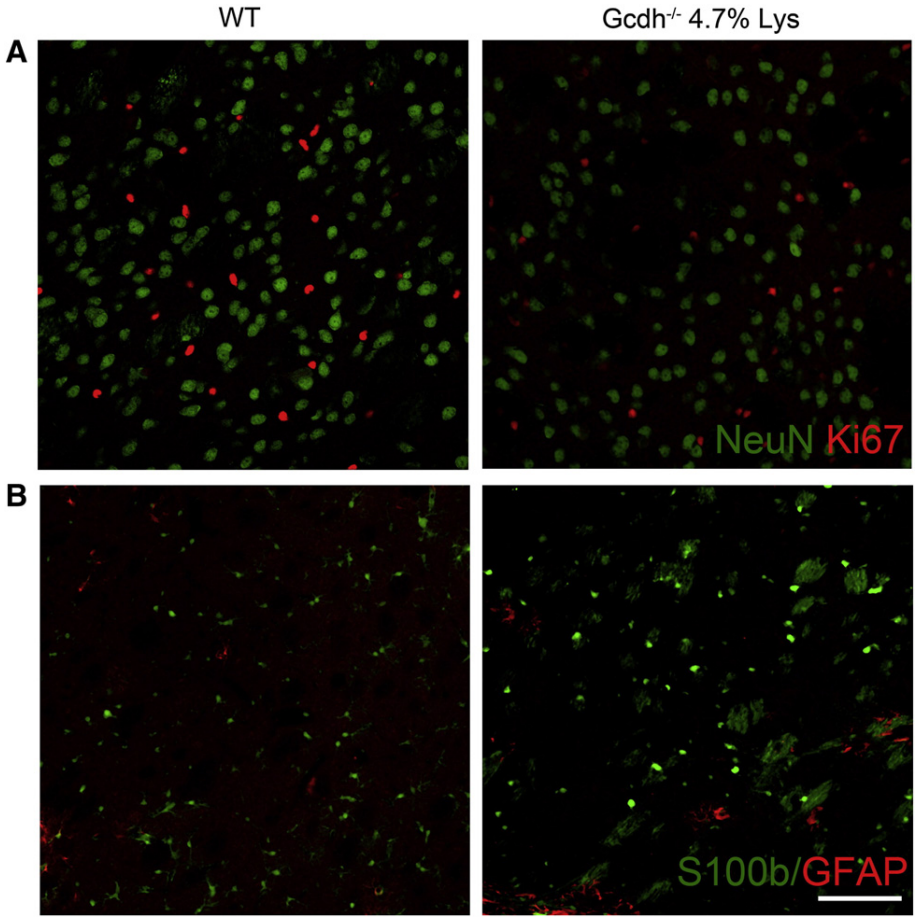


Fig. 7. Histopathological findings in the striatum from the *Gcdh*$^{-/-}$ and WT mice on a high Lys (4.7%) diet. (A) *Gcdh*$^{-/-}$ striatal neuronal NeuN positive population did not suffer significant changes neither in the number nor in the gross immunoreactivity, as compared to WT mice (left panel). Furthermore, Ki67 immunostaining was similar in *Gcdh*$^{-/-}$ and WT mice, discarding a significant injury response. (B) No changes in S100b+ and GFAP+ cell number were observed in high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice, discarding prominent astrogliosis, although a mild increase in GFAP immunoreactivity and an apparent swelling of S100b positive cells were observed in comparison to WT mice (left panel). Calibration bar indicates 50 μm.

induction of lipid peroxidation since malondialdehyde is an end product of membrane fatty acid oxidation [57]. We also observed in *Gcdh*$^{-/-}$ mice fed a high Lys diet a marked increase of DCFH oxidation, which is considered a reliable measurement of reactive species formation [58]. It is therefore presumed that the lipid oxidative damage observed following Lys supplementation could be secondary to the induction of reactive species generation.

Regarding GSH, its levels were significantly reduced in the cerebral cortex and striatum of the *Gcdh*$^{-/-}$ animals that received 2.8% or 4.7% Lys diets. Considering that endogenous GSH, the major naturally-occurring brain antioxidant, is used to evaluate the non-enzymatic antioxidant capacity of a tissue to prevent the damage associated with free radical processes [57], it can be presumed that the antioxidant defenses in the striatum and cerebral cortex of *Gcdh*$^{-/-}$ mice were compromised by Lys enriched diets.

A significant inverse correlation between GSH concentrations and DCFH oxidation (DCF levels) was also demonstrated in the present study, suggesting that the decrease of the brain antioxidant defenses was probably secondary to increased induction of reactive species formation. Therefore, it is feasible that the marked decline of this important cerebral antioxidant defense (GSH) reflects the rapid consumption of this antioxidant by increased reactive species. Otherwise, we cannot exclude the possibility that Lys-induced lipid oxidative damage may have occurred due to GSH depletion.

Moreover, SOD and CAT activities were increased in the cerebral cortex of *Gcdh*$^{-/-}$ animals treated with either 2.8 or 4.7% Lys, with no alterations in the other structures evaluated. In this context, it is widely known that reactive species may upregulate the expression of numerous genes via signaling mechanisms [57,59,60]. Therefore, we presume that overexpression of SOD and CAT at the gene level might have taken place in order to compensate the increased formation of reactive species (superoxide and hydrogen peroxide, respectively), as previously observed in other pathological conditions with oxidative stress [16,61,62].

Our present data indicate that the cerebral cortex and the striatum are more vulnerable to oxidative stress than hippocampus and the peripheral tissues (heart and liver) of *Gcdh*$^{-/-}$ mice submitted to high Lys diet, which mimic the human condition evidencing that these brain regions are more affected and present structural abnormalities in GA I patients.

Interestingly, in a separate set of experiments we compared the oxidative stress parameters in the cerebral cortex and striatum from symptomatic and asymptomatic *Gcdh*$^{-/-}$ mice exposed to 4.7% Lys diet. Symptomatic *Gcdh*$^{-/-}$ mice presented a more accentuated disruption of redox homeostasis, as compared to asymptomatic animals, indicating a probable association between neurologic symptoms and disturbance of redox status.

Since oxidative stress results from an imbalance between the total antioxidant defenses and the reactive species generated in a tissue, our present results strongly indicate that oxidative stress, a deleterious cell condition leading to tissue damage, occurred in *Gcdh*$^{-/-}$ mice submitted to high Lys supplementation [57]. At this point, it

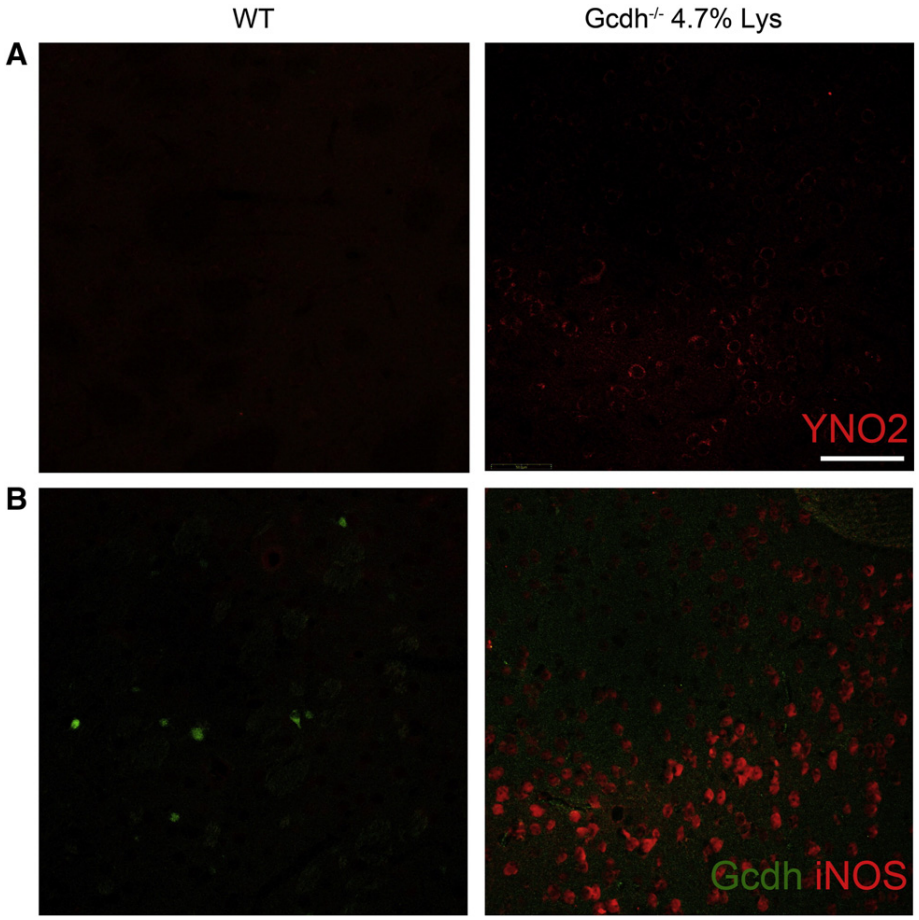


Fig. 8. Oxidative damage markers in the striatum from the *Gcdh*$^{-/-}$ and WT mice on a high Lys diet. (A) Striatal sections from the *Gcdh*$^{-/-}$ mice showed an increased number of nitrotyrosine (YNO2) positive cells and an impressive increase in the number of inducible nitric oxide synthetase (iNOS) (B), as compared to WT mice (left panels). Furthermore, *Gcdh*$^{-/-}$ positive neurons were observed only in WT mice (B). Calibration bar indicates 30 μm.

should be emphasized that the brain has low antioxidant defenses, a high metabolic rate associated with superoxide generation and increased amounts of polyunsaturated fatty acids compared with other tissues, that make this tissue more susceptible to free radical attack [57,63]. 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 [63–71].

On the other hand, it is known that Lys can easily cross the blood–brain barrier in young mice being thereafter converted to GA in the central nervous system [72]. In this scenario, it has been demonstrated that the brain levels of GA increase considerably in *Gcdh*$^{-/-}$ mice supplemented by a high Lys diet [38,39] and this was probably the case in our present study. Taken into consideration these observations and also results from previous studies performed in vitro and in vivo in rat brain showing that GA is able to elicit oxidative stress in this tissue [11,15,26–28], it is conceivable that GA was possibly involved in the disruption of cell redox homeostasis as here observed.

On the other hand, it is unlikely that brain Lys accumulation could be responsible for the results obtained in the present study since it was previously shown that serum and brain Lys levels did not differ between WT, symptomatic and asymptomatic *Gcdh*$^{-/-}$ mice fed a 4.7% Lys supplemented diet [38]. Therefore, distinctly from GA that significantly accumulates in the brain from the knockout mice with high Lys diet, it is presumed that Lys was not responsible to the compromised redox homeostasis observed in the brain of these animals.

Our results on the histopathological findings indicate that the brain of *Gcdh*$^{-/-}$ mice treated with high Lys (4.7%) did not show major structural alterations upon 60 h of treatment. This is interesting and suggests that the significant alterations of oxidative stress parameters were not due to cell death in the central nervous system. On the other hand, we cannot ascertain whether the data described by Zinnanti and collaborators [39] showing an impairment of energy homeostasis paralleled with striatum abnormalities in high Lys (4.7%) -exposed *Gcdh*$^{-/-}$ mice were consequence or cause of brain injury since the biochemical alterations with reduced ATP and phosphocreatine concentrations were obtained during or after neuronal loss and astrogliosis. Furthermore, the increased number of neurons stained with nitrotyrosine (YNO2) and iNOS in striatum of *Gcdh*$^{-/-}$ mice exposed to 4.7% Lys diet reinforces our findings indicating oxidative damage in the brain of these animals. Moreover, the fact that neuronal NeuN staining was weaker and more diffuse and the observations that S100+ cells appeared swollen with increased immunoreactivity in high Lys treated *Gcdh*$^{-/-}$ animals than in WT mice suggest that neural cells may suffer metabolic perturbations previously to lose viability and that oxidative damage may be associated with these findings representing a relevant pathomechanism of ulterior brain injury in the *Gcdh*$^{-/-}$ mice fed a high Lys diet [73]. This is probably the case since symptomatic *Gcdh*$^{-/-}$ mice fed 4.7% Lys presented a more accentuated disruption of redox homeostasis in the cerebral cortex and striatum than asymptomatic *Gcdh*$^{-/-}$ mice, suggesting that oxidative stress was probably associated with the symptoms.

Furthermore, considering that one-fourth of *Gcdh*$^{-/-}$ mice fed a 4.7% Lys supplemented diet present hypoactivity after 48–72 h and die 5 to 7 days later [[], our own observations], whereas the animals with the same genetic background submitted to 2.8% Lys dietary intake are practically asymptomatic for a long time (even 14 days after diet start), it is conceivable that a moderately increased Lys overload (2.8%) may represent a better model of GAI, allowing investigation of underlying mechanisms responsible for brain damage in this disorder.

The present results are in line with the current therapeutic procedure of restricting Lys dietary intake to GA I patients, especially in the first few years of life, where the brain is highly vulnerable to degeneration [3,23]. Furthermore, we have proven that a 3-fold higher Lys diet (2.8%) applied to the genetic mice model established by Koeller and collaborators [37] does not induce death. It seems feasible that this new GA I model using 2.8% Lys-supplemented diet may represent a valuable tool to study long term effects of the putative toxic accumulating metabolites in the central nervous system given the increased number of children who are growing to adolescence and adulthood due to neonatal screening and/or aggressive treatment. The use of this model may be also essential for the development of novel specific therapies.

In conclusion, the present results provide experimental evidence that the cerebral cortex and striatum of 30-day-old *Gcdh*$^{-/-}$ mice submitted to high Lys intake are particularly susceptible to oxidative stress. Furthermore, disruption of redox homeostasis was more evident in symptomatic *Gcdh*$^{-/-}$ mice exposed to a high Lys diet indicating an association between symptoms and alterations of brain redox status in this model of GA I. However, we cannot rule out that other mechanisms such as excitotoxicity, mitochondrial dysfunction and breakdown of the blood–brain barrier [31,33–35,74–77] also contribute in a synergistically way to cause brain damage in GA I.

Conflicts of interest

There are no conflicts of interest between the authors.

Acknowledgments

We are grateful to the financial support of CNPq, PROPESq/UFRGS, FAPERGS, PRONEX, FINEP Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00, and Instituto Nacional de Ciência e Tecnologia-Excitotoxicidade e Neuroproteção (INCT-EN).

References

[1] J. Brismar, P.T. Ozand, CT and MR of the brain in glutaric aciduria type I: a review of 59 published cases and a report of 5 new patients, Am. J. Neuroradiol. 16 (1995) 675–683.
[2] J. Heringer, S.P. Boy, R. Ensenauer, Use of guidelines improves the neurological outcome in glutaric aciduria type I, Ann. Neurol. 68 (2010) 743–752.
[3] K.A. Strauss, D.H. Morton, Type I glutaric aciduria, part 2: a model of acute striatal necrosis, Am. J. Med. Genet. C Semin. Med. Genet. 121C (2003) 53–70.
[4] I. Harting, E. Neumaier-Probst, A. Seitz, E.M. Maier, B. Assmann, I. Baric, M. Troncoso, C. Mühlhausen, J. Zschocke, N.P. Boy, G.F. Hoffmann, S.F. Garbade, S. Kölker, Dynamic changes of striatal and extrastriatal abnormalities in glutaric aciduria type I, Brain 132 (2009) 1764–1782.
[5] K.A. Strauss, J. Lazovic, M. Wintermark, D.H. Morton, Multimodal imaging of striatal degeneration in Amish patients with glutaryl-CoA dehydrogenase deficiency, Brain 130 (2007) 1905–1920.
[6] S.I. Goodman, M.D. Norenberg, R.H. Shikes, D.J. Breslich, P.G. Moe, Glutaric aciduria: biochemical and morphologic considerations, J. Pediatr. 90 (1977) 746–750.
[7] S. Kimura, M. Hara, A. Nezu, H. Osaka, S. Yamazaki, K. Saitoh, Two cases of glutaric aciduria type 1: clinical and neuropathological findings, J. Neurol. Sci. 123 (1994) 38–43.
[8] Y. Mushimoto, S. Fukuda, Y. Hasegawa, Clinical and molecular investigation of 19 Japanese cases of glutaric acidemia type 1, Mol. Genet. Metab. 102 (2011) 343–348.
[9] B. Pérez-Dueñas, A. De La Osa, A. Capdevila, A. Navarro-Sastre, A. Leist, A. Ribes, A. García-Cazorla, M. Serrano, M. Pineda, J. Campistol, Brain injury in glutaric aciduria type I: the value of functional techniques in magnetic resonance imaging, Eur. J. Paediatr. Neurol. 13 (2009) 534–540.
[10] K.B. Dalcin, R.B. Rosa, A.L. Schmidt, J.S. Winter, G. Leipnitz, C.S. Dutra-Filho, C.M. Wannmacher, L.O. Porciúncula, D.O. Souza, M. Wajner, Age and brain structural related effects of glutaric and 3-hydroxyglutaric acids on glutamate binding to plasma membranes during rat brain development, Cell. Mol. Neurobiol. 27 (2007) 805–818.
[11] F. de Oliveira Marques, M.E. Hagen, C.D. Pederzolli, A.M. Sgaravatti, K. Durigon, C.G. Testa, C.M. Wannmacher, A.T. de Souza Wyse, M. Wajner, C.S. Dutra-Filho, Glutaric acid induces oxidative stress in brain of young rats, Brain Res. 964 (2003) 153–158.
[12] G.C. Ferreira, C.M. Viegas, P.F. Schuck, A. Tonin, C.A.J. Ribeiro, D.M. Coelho, T. Dalla-Costa, A. Latini, A.T. Wyse, C.M. Wannmacher, C.R. Vargas, M. Wajner, Glutaric acid administration impairs energy metabolism in midbrain midbrain and skeletal muscle of young rats, Neurochem. Res. 30 (2005) 1123–1131.
[13] G.C. Ferreira, A. Tonin, P.F. Schuck, C.M. Viegas, P.C. Ceolato, A. Latini, M.L. Perry, A.T. Wyse, C.S. Dutra-Filho, C.M. Wannmacher, C.R. Vargas, M. Wajner, Evidence for a synergistic action of glutaric and 3-hydroxyglutaric acids disturbing rat brain energy metabolism, Int. J. Dev. Neurosci. 25 (2007) 391–398.
[14] G.C. Ferreira, P.F. Schuck, C.M. Viegas, A. Tonin, A. Latini, C.S. Dutra-Filho, A.T. Wyse, C.M. Wannmacher, C.R. Vargas, M. Wajner, Energy metabolism is compromised in skeletal muscle of rats chronically-treated with glutaric acid, Metab. Brain Dis. 22 (2007) 111–123.
[15] M.R. Fighera, L.F. Royes, A.F. Furian, M.S. Oliveira, N.G. Fiorenza, R. Frussa-Filho, J.C. Petry, R.C. Coelho, C.F. Mello, GM1 ganglioside prevents seizures, Na^+, K^+-ATPase activity inhibition and oxidative stress induced by glutaric acid and pentylenetetrazole, Neurobiol. Dis. 22 (2006) 611–623.
[16] P. Jafari, O. Braissant, L. Bonafé, D. Ballhausen, The unsolved puzzle of neuropathogenesis in glutaric aciduria type I, Mol. Genet. Metab. 104 (2011) 425–437.
[17] S. Kölker, B. Ahlemeyer, J. Krieglstein, G.F. Hoffmann, 3-Hydroxyglutaric and glutaric acids are neurotoxic through NMDA receptors in vitro, J. Inherit. Metab. Dis. 22 (1999) 259–262.
[18] S. Kölker, B. Ahlemeyer, J. Krieglstein, G.F. Hoffmann, Contribution of reactive oxygen species to 3-hydroxyglutarate neurotoxicity in primary neuronal cultures from chick embryo telencephalons, Pediatr. Res. 50 (2001) 76–82.
[19] S. Kölker, B. Ahlemeyer, R. Hühne, E. Mayatepek, J. Krieglstein, G.F. Hoffmann, Potentiation of 3-hydroxyglutarate neurotoxicity following induction of astrocytic iNOS in neonatal rat hippocampal cultures, Eur. J. Neurosci. 13 (2001) 2115–2122.
[20] S. Kölker, J.G. Okun, B. Ahlemeyer, A.T. Wyse, F. Hörster, M. Wajner, D. Kohlmüller, E. Mayatepek, J. Krieglstein, G.F. Hoffmann, Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons, J. Neurosci. Res. 68 (2002) 424–431.
[21] S. Kölker, G. Köhr, B. Ahlemeyer, J.G. Okun, V. Pawlak, F. Hörster, E. Mayatepek, J. Krieglstein, G.F. Hoffmann, Ca(2+) and Na(+) dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons, Pediatr. Res. 52 (2002) 199–206.
[22] S. Kölker, D.M. Koeller, S. Sauer, F. Hörster, M.A. Schwab, G.F. Hoffmann, K. Ullrich, J.G. Okun, Excitotoxicity and bioenergetics in glutaryl-CoA dehydrogenase deficiency, J. Inherit. Metab. Dis. 27 (2004) 805–812.
[23] S. Kölker, S.W. Sauer, J.G. Okun, G.F. Hoffmann, D.M. Koeller, Lysine intake and neurotoxicity in glutaric aciduria type I: towards a rationale for therapy? Brain 129 (2006) e54.
[24] A. Latini, R. Borba Rosa, K. Scussiato, S. Llesuy, A. Belló-Klein, M. Wajner, 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats, Brain Res. 956 (2002) 367–373.
[25] A. Latini, K. Scussiato, G. Leipnitz, C.S. Dutra-Filho, M. Wajner, Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum, J. Inherit. Metab. Dis. 28 (2005) 57–67.
[26] A. Latini, G.C. Ferreira, K. Scussiato, P.F. Schuck, A.F. Solano, C.S. Dutra-Filho, C.R. Vargas, M. Wajner, Induction of oxidative stress by chronic and acute glutaric acid administration to rats, Cell. Mol. Neurobiol. 27 (2007) 423–438.
[27] D.V. Magni, A.F. Furian, M.S. Oliveira, M.A. Souza, F. Lunardi, J. Ferreira, C.F. Mello, L.F. Royes, M.R. Fighera, Kinetic characterization of l-[(3)H]glutamate uptake inhibition and increase oxidative damage induced by glutaric acid in striatal synaptosomes of rats, Int. J. Dev. Neurosci. 27 (2009) 65–72.
[28] S. Olivera, A. Fernandez, A. Latini, J.C. Rosillo, G. Casanova, M. Wajner, P. Cassina, L. Barbeito, Astrocytic proliferation and mitochondrial dysfunction induced by accumulated glutaric acidemia I (GAI) metabolites: possible implications for GAI pathogenesis, Neurobiol. Dis. 32 (2008) 528–534.
[29] S. Olivera-Bravo, A. Fernández, M.N. Sarlabós, J.C. Rosillo, G. Casanova, M. Jiménez, L. Barbeito, Neonatal astrocyte damage is sufficient to trigger progressive striatal degeneration in a rat model of glutaric acidemia-I, PLoS One 6 (2011) e20831.
[30] L.O. Porciúncula, A. Dal-Pizzol Jr., A.S. Coitinho, T. Emanuelli, D.O. Souza, M. Wajner, Inhibition of synaptosomal [3H]glutamate uptake and [3H]glutamate binding to plasma membranes from brain of young rats by glutaric acid in vitro, J. Neurol. Sci. 173 (2000) 93–96.
[31] L.O. Porciúncula, T. Emanuelli, R.G. Tavares, C. Schwarzbold, M.E. Frizzo, D.O. Souza, M. Wajner, Glutaric acid stimulates glutamate binding and astrocytic uptake and inhibits vesicular glutamate uptake in forebrain from young rats, Neurochem. Int. 45 (2004) 1075–1086.
[32] R.B. Rosa, C. Schwarzbold, K.B. Dalcin, G.C. Ghisleni, C.A. Ribeiro, M.B. Moretto, M.E. Frizzo, G.F. Hoffmann, D.O. Souza, M. Wajner, Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats, Neurochem. Int. 45 (2004) 1087–1094.

[33] R.B. Rosa, K.B. Dalcin, A.L. Schmidt, D. Gerhardt, C.A. Ribeiro, G.C. Ferreira, P.F. Schuck, A.T. Wyse, L.O. Porciúncula, S. Wofchuk, C.G. Salbego, D.O. Souza, M. Wajner, Evidence that glutaric acid reduces glutamate uptake by cerebral cortex of infant rats, Life Sci. 81 (2007) 1668–1676.
[34] O. Stokke, S.I. Goodman, P.G. Moe, Inhibition of brain glutamate decarboxylase by glutarate, glutaconate, and beta-hydroxyglutarate: explanation of the symptoms in glutaric aciduria? Clin. Chim. Acta 66 (1976) 411–415.
[35] M. Wajner, S. Kölker, D.O. Souza, G.F. Hoffmann, C.F. de Mello, Modulation of glutamatergic and GABAergic neurotransmission in glutaryl-CoA dehydrogenase deficiency, J. Inherit. Metab. Dis. 27 (2004) 825–828.
[36] C.B. Funk, A.N. Prasad, M.R. Del Bigio, Preliminary attempts to establish a rat model of striatal injury in glutaric acidaemia type I, J. Inherit. Metab. Dis. 27 (2004) 819–824.
[37] D.M. Koeller, M. Woontner, L.S. Crnic, B. Kleinschmidt-DeMasters, J. Stephens, E.L. Hunt, S.I. Goodman, Biochemical, pathologic and behavioral analysis of a mouse model of glutaric acidemia type I, Hum. Mol. Genet. 11 (2002) 347–357.
[38] W.J. Zinnanti, J. Lazovic, E.B. Wolpert, D.A. Antonetti, M.B. Smith, J.R. Connor, M. Woontner, S.I. Goodman, K.C. Cheng, A diet-induced mouse model for glutaric aciduria type I, Brain 129 (2006) 899–910.
[39] W.J. Zinnanti, J. Lazovic, C. Housman, Mechanism of age-dependent susceptibility and novel treatment strategy in glutaric acidemia type I, J. Clin. Invest. 117 (2007) 3258–3270.
[40] B. Seminotti, M.S. da Rosa, C.G. Fernandes, A.U. Amaral, L.M. Braga, G. Leipnitz, D.O.G. de Souza, M. Woontner, D.M. Koeller, S. Goodman, M. Wajner, Induction of oxidative stress in brain of glutaryl-CoA dehydrogenase deficient mice by acute lysine administration, Mol. Genet. Metab. 106 (2012) 31–38.
[41] P. Evelson, M. Travacio, M. Repetto, J. Escobar, S. Llesuy, E.A. Lissi, Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols, Arch. Biochem. Biophys. 388 (2001) 261–266.
[42] S.L. Arnaiz, S. Llesuy, Oxidative stress in mouse heart by antitumoral drugs: a comparative study of doxorubicin and mitoxantrone, Toxicology 77 (1993) 31–38.
[43] E. Cadenas, A.L. Varsavsky, A. Boveris, B. Chance, Oxygen- or organic hydroperoxide-induced chemiluminescence of brain and liver homogenates, Biochem. J. 198 (1981) 645–654.
[44] B. Gonzalez Flecha, S. Llesuy, A. Boveris, Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver, and muscle, Free Radic. Biol. Med. 10 (1991) 93–100.
[45] S. Llesuy, P. Evelson, B. Gonzalez-Flecha, J. Peralta, M.C. Carreras, J.J. Poderoso, A. Boveris, Oxidative stress in muscle and liver of rats with septic syndrome, Free Radic. Biol. Med. 16 (1994) 445–451.
[46] S.L. Rutledge, G.T. Berry, C.A. Stanley, J.L.K. van Hove, D. Millington, Glycine and L-carnitine therapy in 3-methylcrotonyl-CoA carboxylase deficiency, J. Inherit. Metab. Dis. 18 (1995) 299–305.
[47] M.T. Dresch, S.B. Rossato, V.D. Kappel, R. Biegelmeyer, M.L. Hoff, P. Mayorga, J.A. Zuanazzi, A.T. Henriques, J.C. Moreira, Optimization and validation of an alternative method to evaluate total reactive antioxidant potential, Anal. Biochem. 385 (2009) 107–114.
[48] J. Stocks, J.M. Gutteridge, R.J. Sharp, T.L. Dormandy, Assay using brain homogenate for measuring the antioxidant activity of biological fluids, Clin. Sci. Mol. Med. 47 (1974) 215–222.
[49] K. Yagi, Simple procedure for specific assay of lipid hydroperoxides in serum or plasma, Methods Mol. Biol. 108 (1998) 107–110.
[50] C.P. LeBel, H. Ischiropoulos, S.C. Bondy, Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress, Chem. Res. Toxicol. 5 (1992) 227–231.
[51] R.W. Browne, D. Armstrong, Reduced glutathione and glutathione disulfide, Methods Mol. Biol. 108 (1998) 347–352.
[52] A. Wendel, Glutathione peroxidase, Methods Enzymol. 77 (1981) 325–332.
[53] S.L. Marklund, Pyrogallol autoxidation, in: Handbook for Oxygen Radical Research, CRC Press, Boca Raton, FL, 1985, pp. 243–247.
[54] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.
[55] S.F. Leong, J.B. Clark, Regional development of glutamate dehydrogenase in the rat brain, J. Neurochem. 43 (1984) 106–111.
[56] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[57] B. Halliwell, J.M.C. Gutteridge, Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Oxford University Press Inc., Oxford, 2007, pp. 187–340.
[58] P. Bilski, A.G. Belanger, C.F. Chignell, Photosensitized oxidation of 2′,7′-dichlorofluorescin: singlet oxygen does not contribute to the formation of fluorescent oxidation product 2′,7′-dichlorofluorescein, Free Radic. Biol. Med. 33 (2002) 938–946.
[59] V. Lakshminarayanan, E.A. Drab-Weiss, K.A. Roebuck, H_2O_2 and tumor necrosis factor-alpha induce differential binding of the redox-responsive transcription factors AP-1 and NF-kappaB to the interleukin-8 promoter in endothelial and epithelial cells, J. Biol. Chem. 273 (1998) 32670–32678.
[60] T.H. Rushmore, M.R. Morton, C.B. Pickett, The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity, J. Biol. Chem. 266 (1991) 11632–11639.
[61] S. Genet, K.R. Kale, N.Z. Baquer, Alterations in antioxidant enzymes and oxidative damage in experimental diabetic rat tissues: effect of vanadate and fenugreek (*Trigonella foenum graecum*), Mol. Cell. Biochem. 236 (2002) 7–12.
[62] S. Kaushik, J. Kauer, Chronic cold exposure affects the antioxidant defense system in various rat tissues, Clin. Chim. Acta 333 (2003) 69–77.
[63] B. Halliwell, J.M.C. Gutteridge, Oxygen radicals and nervous system, Trends Neurosci. 8 (1996) 22–26.
[64] C. Behl, B. Moosmann, Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach, Free Radic. Biol. Med. 33 (2002) 182–191.
[65] D. Berg, M.B. Youdim, Role of iron in neurodegenerative disorders, Top. Magn. Reson. Imaging 17 (2006) 5–17.
[66] M.B. Bogdanov, O.A. Andreassen, A. Dedeoglu, R.J. Ferrante, M.F. Beal, Increased oxidative damage to DNA in a transgenic mouse model of Huntington's disease, J. Neurochem. 79 (2001) 1246–1249.
[67] E. Karelson, N. Bogdanovic, A. Garlind, B. Winblad, K. Zilmer, T. Kullisaar, T. Vihalemm, C. Kairane, M. Zilmer, The cerebrocortical areas in normal brain aging and in Alzheimer's disease: noticeable differences in the lipid peroxidation level and in antioxidant defense, Neurochem. Res. 26 (2001) 353–361.
[68] M. Mancuso, F. Coppede, L. Migliore, G. Siciliano, L. Murri, Mitochondrial dysfunction, oxidative stress and neurodegeneration, J. Alzheimers Dis. 10 (2006) 59–73.
[69] E. Méndez-Alvarez, R. Soto-Otero, A. Hermida-Ameijeiras, M.E. López-Martín, J.L. Labandeira-García, Effect of iron and manganese on hydroxyl radical production by 6-hydroxydopamine: mediation of antioxidants, Free Radic. Biol. Med. 31 (2001) 986–998.
[70] F. Pérez-Severiano, C. Ríos, J. Segovia, Striatal oxidative damage parallels the expression of a neurological phenotype in mice transgenic for the mutation of Huntington's disease, Brain Res. 862 (2000) 234–237.
[71] N. Stoy, G.M. Mackay, C.M. Forrest, J. Christofides, M. Egerton, T.W. Stone, L.G. Darlington, Tryptophan metabolism and oxidative stress in patients with Huntington's disease, J. Neurochem. 93 (2005) 611–623.
[72] Q.R. Smith, J.S. Stoll, Blood–brain barrier amino acid transport, in: W.M. Pardridge (Ed.), Introduction to the Blood–Brain Barrier, Cambridge University Press, Cambridge, 1998, pp. 188–197.
[73] I. Unal-Cevik, M. Kilinç, Y. Gürsoy-Ozdemir, G. Gurer, T. Dalkara, Loss of NeuN immunoreactivity after cerebral ischemia does not indicate neuronal cell loss: a cautionary note, Brain Res. 1015 (2004) 169–174.
[74] S. Kölker, B. Ahlemeyer, J. Krieglstein, G.F. Hoffmann, Evaluation of trigger factors of acute encephalopathy in glutaric aciduria type I: fever and tumour necrosis factor-alpha, J. Inherit. Metab. Dis. 23 (2000) 359–362.
[75] S.W. Sauer, J.G. Okun, G. Fricker, A. Mahringer, I. Müller, L.R. Crnic, C. Mühlhausen, G.F. Hoffmann, F. Hörster, S.I. Goodman, C.O. Harding, D.M. Koeller, S. Kölker, 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 (2006) 899–910.
[76] K.A. Strauss, P. Donnelly, M. Wintermark, Cerebral haemodynamics in patients with glutaryl-coenzyme A dehydrogenase deficiency, Brain 133 (2010) 76–92.
[77] K.A. Strauss, J. Brumbaugh, A. Duffy, B. Wardley, D. Robinson, C. Hendrickson, S. Tortorelli, A.B. Moser, E.G. Puffenberger, N.L. Rider, D.H. Morton, Safety, efficacy and physiological actions of a lysine-free, arginine-rich formula to treat glutaryl-CoA dehydrogenase deficiency: focus on cerebral amino acid influx, Mol. Genet. Metab. 104 (2011) 93–106.

Capítulo IV

Avaliação de parâmetros de estresse oxidativo e análise histopatológica em córtex cerebral e estriado de camundongos nocaute para a enzima glutaril-CoA desidrogenase submetidos à dieta crônica com lisina

1.INTRODUÇÃO

A acidemia glutárica tipo I (AG I) é uma doença neurometabólica autossômica recessiva causada pela deficiência da enzima glutaril-CoA desidrogenase (GCDH), que participa da rota de catabolismo dos aminoácidos lisina (Lis), hidroxilisina e triptofano. O bloqueio dessa atividade enzimática na AG I leva ao acúmulo predominante dos ácidos glutárico (AG) e 3-hidroxiglutárico (3HG) nos tecidos e líquidos biológicos (sangue, urina e líquor) dos pacientes afetados. A prevalência da doença é estimada em 1: 30.000-1:100.000 nascidos vivos, podendo atingir uma alta prevalência (até 1: 300 nascidos vivos) em algumas comunidades fechadas (Goodman *et al.*, 2001; Kölker *et al.*, 2007). A acidemia glutárica tipo I (AG I) é considerada uma acidemia orgânica "cerebral", pois os pacientes apresentam essencialmente sintomatologia neurológica. Ao nascimento, alguns pacientes apresentam macrocefalia e atrofia cortical frontotemporal, enquanto outros se desenvolvem normalmente. Entre 3 e 36 meses de idade, uma característica degeneração bilateral aguda do estriado acomete os pacientes durante crises encefalopáticas geralmente precipitadas por infecções ou vacinações (situações em que o paciente se encontra em catabolismo elevado). Após essas crises agudas, há o surgimento de sintomas relacionados à destruição estriatal, tais como distonia e discinesia, hipotonia, convulsões, rigidez muscular e espasticidade (Goodman *et al.*, 2001; Hoffmann e Zschocke, 1999; Kölker *et al.*, 2008). Tal fato sugere uma "janela de vulnerabilidade" para o aparecimento dos sintomas, provavelmente relacionada ao período de desenvolvimento cerebral.

Apesar de algumas investigações prévias terem sugerido efeitos tóxicos do AG e do 3HG, até o presente momento os mecanismos fisiopatológicos que levam ao dano cortical e estriatal apresentado pelos pacientes afetados pela AG I ainda não foram totalmente elucidados.

Diversos autores relacionam as vacuolizações encontradas em estudos *postmortem* de cérebro de pacientes afetados pela deficiência da GCDH à excitotoxicidade (Goodman *et al.*, 1977; Hoffmann e Zschocke, 1999) e conseqüente dano celular (Danbolt, 2001). Esta hipótese está baseada na similaridade estrutural existente entre o glutamato e os principais ácidos orgânicos (AG e 3HG) acumulados na AG I (Flott-Rahmel *et al.*, 1997; Wajner *et al.*, 2004). Nesse sentido, vários trabalhos tentam explicar a neurotoxicidade da AG I pela interação desses ácidos orgânicos com receptores e transportadores glutamatérgicos (Bjugstad *et al.*, 2001; Dalcin *et al*., 2007; Kölker *et al.*, 2008; Porciúncula *et al.*, 2000; Porciúncula *et al.*, 2004; Rosa *et al*., 2004; Wajner et al., 2004). No entanto, apesar de diversas evidências da neurotoxicidade destas substâncias relacionadas ao sistema glutamatérgico, recentes trabalhos não confirmam essa hipótese (Freudenberg *et al*., 2004; Lund *et al.*, 2004), fazendo com que esta questão continue sob intenso debate.

Recentemente, o modelo nocaute para o gene da GCDH (*Gcdh*$^{-/-}$) em camundongos (Köeller *et al.*, 2002) foi aperfeiçoado por Zinnanti e colaboradores (2006) com a administração, via oral, de uma sobrecarga de Lis aos animais. Neste particular, foi verificado que as concentrações de AG no cérebro dos camundongos *Gcdh*$^{-/-}$ aumentaram significativamente e que os mesmos apresentaram um padrão de neurodegeneração dependente do

estágio de desenvolvimento, semelhante ao apresentado pelos pacientes afetados pela AG I (lesão estriatal aguda e leucodistrofia progressiva), além de provocar a perda de seletividade da barreira hematoencefálica. Os mesmos autores demonstraram que essa sobrecarga de lisina provoca uma disfunção mitocondrial, e propuseram a utilização da suplementação com glicose e homoarginina para reduzir o acúmulo cerebral dos metabólitos tóxicos gerados pela deficiência da GCDH (Zinnanti *et al.*, 2007). Apesar da intensa investigação, as causas da suscetibilidade frontotemporal cortical durante a gestação e da janela de vulnerabilidade estriatal durante os primeiros anos de vida permanecem obscuras, constituindo-se nos principais desafios da pesquisa da patogênese da AG I (Goodman, 2004).

Nosso propósito na presente investigação foi avaliar o efeito de uma dieta crônica com altas concentrações de lisina (2,8 %) (40 dias de dieta a partir do 21º. dia de vida) sobre a oxidação de lipídios (medida de substâncias reativas ao ácido tiobarbitúrico - TBA-RS), bem como sobre as defesas antioxidantes (concentrações de glutationa reduzida – GSH - e as atividades das enzimas antioxidantes glutationa peroxidase –GPx -, superóxido dismutase (SOD), catalase (CAT), glutationa redutase (GR) e glicose-6-fosfato desidrogenase , G6PDH) em córtex cerebral e estriado de animais selvagens (*Gcdh*$^{+/+}$) e *Gcdh*$^{-/-}$.

2. MATERIAL E MÉTODOS

2.1. Animais

Camundongos previamente genotipados de 60 dias de vida foram usados para a avaliação dos parâmetros de estresse oxidativo e viabilidade e morfologia celular nas várias estruturas cerebrais. Os animais nocaute (*Gcdh* $^{-/-}$) e selvagens (*Gcdh*$^{+/+}$) foram submetidos à dieta com 0,9 % ou 2,8 % de lisina (Lis) por 40 dias a partir do 21º dia de idade (tratamento crônico).

2.1.1. Manutenção da colônia

Os camundongos da linhagem 129SvEv foram mantidos na Unidade de Experimentação Animal do Hospital de Clínicas de Porto Alegre (UEA-HCPA).

O acasalamento foi feito através de camundongos heterozigotos e homozigotos, já que os homozigotos doentes são férteis. A prole foi testada por métodos de biologia molecular no desmame, momento em que os animais foram marcados pelo método de perfurações no pavilhão auricular. Um número máximo de 8 filhotes foi mantido por caixa, sendo dada preferência aos homozigotos afetados (para uso nos experimentos) e heterozigotos (para manutenção da colônia). Os filhotes excedentes foram sacrificados. Esses animais foram mantidos com a mãe até o desmame e posteriormente utilizados em outros experimentos. Aos 21 dias, os animais foram desmamados e mantidos em gaiolas separados por sexo até o momento dos experimentos. Camundongos selvagens da mesma linhagem de mesma idade foram utilizados como controle.

Os animais foram mantidos em gaiolas plásticas em ambiente controlado com ciclos de 12 h de luz e escuridão na UEA-HCPA. Os animais tiveram livre acesso a água e ração padrão (0,9 % Lis) ou com sobrecarga de Lis na concentração de 2,8 %.

2.2. Preparação das amostras para técnicas de estresse oxidativo

Os animais foram sacrificados por decapitação sem anestesia aos 60 dias de vida, o cérebro foi imediatamente separado e as estruturas córtex cerebral e estriado isoladas em placa de Petri colocada sobre gelo. Homogeneizados foram preparadas de cada estrutura cerebral. Após, foram retiradas alíquotas para a realização das técnicas bioquímicas.

2.3. Preparação de sobrenadantes das estruturas cerebrais

Após a dissecação, as estruturas foram pesadas e homogeneizadas em tampão fosfato de sódio 20 mM contendo cloreto de potássio 140 mM, pH 7,4, na proporção de 1:10 (peso / volume) e o homogeneizado obtido foi levado à centrifugação a 750 *g* durante 10 min a 4 ºC. O sobrenadante foi empregado para a medida dos diferentes parâmetros de estresse oxidativo.

2.4. Parâmetros de estresse oxidativo

2.4.1. Avaliação da peroxidação lipídica

A avaliação de dano oxidativo lipídico foi feita através da medida dos níveis de substâncias reativas ao ácido tiobarbitúrico (TBA-RS) e foi realizada de acordo com o método de Yagi (1998). Foram adicionados a 100 µL de amostra 200 µL de ácido tricloroacético 10 % e 300 µL de ácido tiobarbitúrico

0,67%. A mistura foi levada a um banho fervente durante 2 h e após resfriada em água à temperatura ambiente. Foram adicionados 400 µL de butanol, os tubos foram agitados por exatos 20 segundos e centrifugados a 5.000 x *g* durante 3 minutos. Após a centrifugação, foram retirados 300 µL da fase superior (fase orgânica), os quais foram lidos espectrofluorimetricamente em comprimentos de onda de excitação e emissão de 515 nm e 553 nm, respectivamente. Concomitantemente, foi feita uma curva de calibração com 1, 1, 3, 3-tetrametoxipropano, na qual todos os pontos foram tratados da mesma forma que as amostras. Os resultados foram calculados como nmol TBA-RS / mg de proteína.

2.4.2. Medida das concentrações de glutationa reduzida (GSH)

As concentrações GSH foram medidas de acordo com a técnica de Browne & Armstrong (1998). Uma alíquota de 150 µL foi tratada com ácido metafosfórico com o objetivo de precipitar as proteínas e proporcionar um meio com pH ácido para a GSH ser mantida na forma reduzida. Após centrifugação, foram adicionados 15 µL de *o*-ftaldialdeído e 255 µL de tampão a 30 µL do sobrenadante obtido. A fluorescência gerada foi medida com comprimentos de onda de excitação e emissão de 350 e 420 nm, respectivamente. A curva de calibração foi realizada com GSH padrão (0,001-1 mM) e os resultados foram calculados como GSH / mg de proteína.

2.4.3. Medida da atividade da glutationa peroxidase (GPx)

A atividade da GPx foi determinada pelo método de Wendel (1981) usando hidroperóxido de tert-butila como substrato. Os seguintes reagentes

foram adicionados: tampão fosfato de potássio 100 mM, pH 7,0, contendo EDTA 1 mM, azida sódica 40 mM, glutationa 100 mM, glutationa redutase 10 U / ml, NADPH 10 mM e a amostra (0,1 – 0,3 µg de proteína). Esta mistura foi incubada a 25 ^{0}C durante 1 minuto, a fim de estabilizar o meio, e após foi adicionado hidroperóxido de tert-butila 10 mM para iniciar a reação. A queda da absorvância a 340 nm foi acompanhada durante 240 segundos e a atividade da GPx foi calculada utilizando-se o coeficiente de extinção do NADPH a 340 nm de 6,2 mM^{-1} cm^{-1}. A atividade da GPx foi calculada como U / mg de proteína (1U = 1 µmol NADPH consumido / min).

2.4.4. Medida da atividade da superóxido dismutase (SOD)

A atividade da SOD, de acordo com Marklund (1985), foi avaliada baseando-se na capacidade do piragalol de se autoxidar em um processo altamente dependente de O2•-, que é um substrato para a SOD. A inibição da autooxidação desse composto ocorre na presença da SOD, cuja atividade pode ser indiretamente medida no espectrofotômetro à 420 nm. O meio de reação consiste em tampão Tris 50 mM, ácido etilenodiaminotetracético, pH 8,2, catalase 80 U / mL, piragalol 0,38 mM e aproximadamente 1 µg de proteína da amostra. Uma curva de calibração foi feita com SOD purificada como padrão para calcular a atividade da SOD presente nas amostras. Os resultados foram expressos como U / mg de proteína.

2.4.5. Medida da atividade catalase (CAT)

A atividade desta enzima foi realizada através do método de Aebi (1984). Em 100 µL do sobrenadante adicinou-se 10 µl de Triton 0,1 %

seguido de agitação, e essa mistura foi conservada em gelo durante 15 minutos. Em 50 mL de tampão fosfato de potássio 10 mM, pH 7,0 foram adicionados 100 µl de H_2O_2 30% (v/v) e à esse meio adicinou-se 25 µl de amostra. A leitura da queda da absorvância do H_2O_2 em espectrofotômetro a 240 nm, à temperatura ambiente (22 ºC ± 2) foi feita por durante 100 segundos. Para o cálculo da CAT o coeficiente de extinção do H_2O_2 de 43.6 $mM^{-1}cm^{-1}$ foi usado. Os resultados da atividade da CAT foram expressos em U / mg proteína (1U =1 µmol H_2O_2 consumido / min.

2.4.6. Medida da atividade da glutationa redutase (GR)

A atividade da GR foi avaliada de acordo com Calberg e Manervik (1985), usando glutationa oxidada (GSSG) e NADPH como substratos. Determinou-se a atividade enzimática monitorando o desaparecimento de NADPH a 349 nm num meio contendo tampão fosfato de sódio 200 mM, pH 7,5, contendo ácido etilenediaminotetracético 6,3 mM, GSSG 1 mM, NADPH 0,1 mM e amostra com aproximadamente 3 µg de proteína. Uma unidade de GR (U) é definida como 1 µmol de GSSG reduzida por minuto. A atividade específica foi calculada como U / mg de proteína.

2.4.7. Medida da atividade da glicose-6-fosfato desidrogenase (G6PDH)

A atividade da G6PDH foi realizada através do método descrito por Leong e Clark (55), com uma mistura contendo Tris-HCl 100mM pH 7,5, $MgCl_2$ 10mM, NADP+ 0,5mM e sobrenadante (aproximadamente 3 µg de proteína). A reação foi iniciada com a adição de glicose-6-fosfato 1mM e acompanhada em espectrofotômetro a 340 nm. Uma unidade de G6PDH

corresponde a 1mmol de substrato transformado por minuto, e atividade foi calculada como U / mg de proteína.

2.5. Análise histológica

Para as análises histológicas, os animais foram anestesiados, mortos e o cérebro foi removido e colocado em formaldeído 10 % por 24 horas. Após, os mesmos foram emblocados em parafina, e seções coronais (3 μm) foram preparadas em micrótomo. Lâminas coradas com hematoxilina e eosina foram preparadas, e imagens do córtex cerebral e do estriado foram obtidas através de um microscópio óptico.

2.6. Dosagem de proteínas

A dosagem de proteínas nas amostras foi realizada através do método de Lowry (1951), usando albumina sérica bovina como padrão.

2.7. Análise estatística

As análises estatísticas foram feitas pelo teste *t* de Student para amostras independentes, e através do programa SPSS versão 15.0. Diferenças significativas foram consideradas quando $P < 0,05$.

3. RESULTADOS

3.1. Efeito da dieta crônica de lisina sobre a medida de substâncias reativas ao ácido tiobarbitúrico (TBA-RS) em córtex cerebral e estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ de 60 dias de vida

Inicialmente, estudamos o efeito da dieta crônica de Lis 2,8% por 40 dias sobre a medida de substâncias reativas ao ácido tiobarbitúrico (TBA-RS) em córtex cerebral e estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ de 60 dias de vida. Observamos que a dieta alterou significativamente os níveis de TBA-RS nas duas estruturas estudadas (Figura 3.1 A e B).

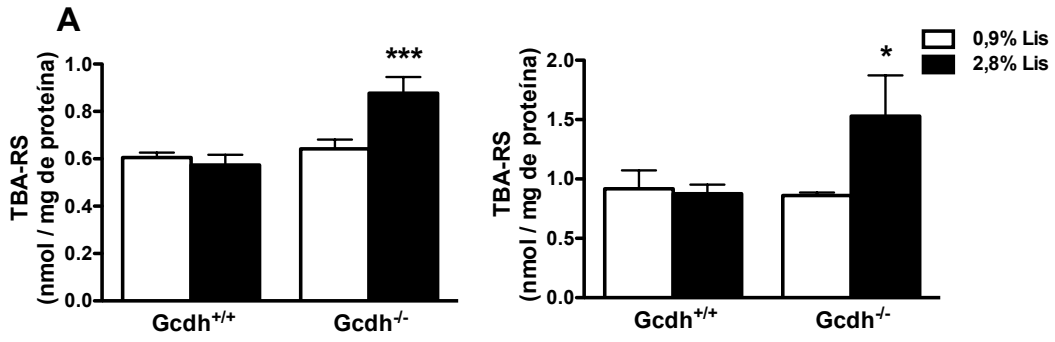


Figura 3.1 – Efeito da dieta crônica de lisina 2,8% sobre os níveis de substâncias reativas ao ácido tiobarbitúrico (TBA-RS) em sobrenadante de córtex cerebral e estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$. Os valores representam média ± desvio padrão da média (n=5). Os resultados foram analisados por teste *t* de Student para amostras independentes. * $P < 0,05$; *** $P < 0,001$.

3.2. Efeito da dieta crônica de lisina sobre a medida as concentrações de glutationa reduzida (GSH) em córtex cerebral e estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ de 60 dias de vida

A figura 3.2 mostra que a administração de uma dieta enriquecida em Lis não alterou as concentrações de GSH 40 dias após o início da dieta.

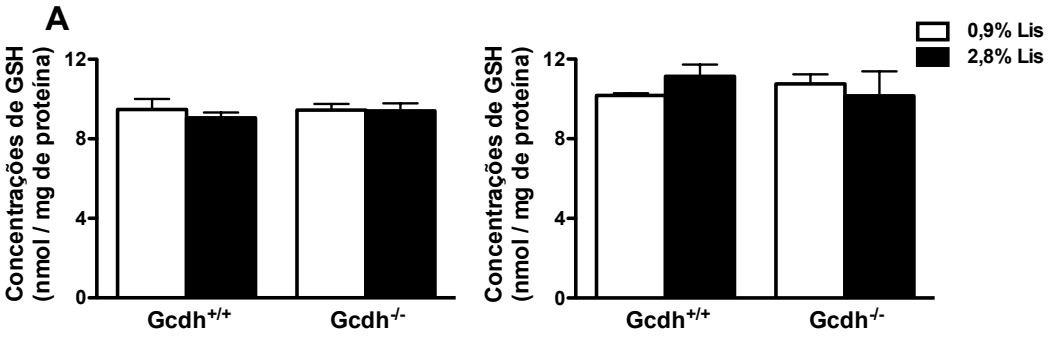


Figura 3.2 – Efeito da dieta crônica de lisina 2,8% sobre as concentrações de glutationa reduzida (GSH) em sobrenadante de córtex cerebral e estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$. Os valores representam média ± desvio padrão da média (n=5). Os resultados foram analisados por teste *t* de Student para amostras independentes. Não houve diferença significativa entre os grupos.

3.3. Efeito da dieta crônica de lisina sobre a atividade das enzimas antioxidantes glutationa peroxidase (GPx), superóxido dismutase (SOD), catalase (CAT), glutationa redutase (GR) e glicose-6-fosfato desidrogenase (G6PDH) em córtex cerebral e estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ de 60 dias de vida

As atividades das enzimas antioxidantes não foram alteradas pela administração de uma dieta enriquecida em Lis, em córtex cerebral (Figura 3.3.1) e estriado dos animais *Gcdh*$^{-/-}$ (Figura 3.3.2).

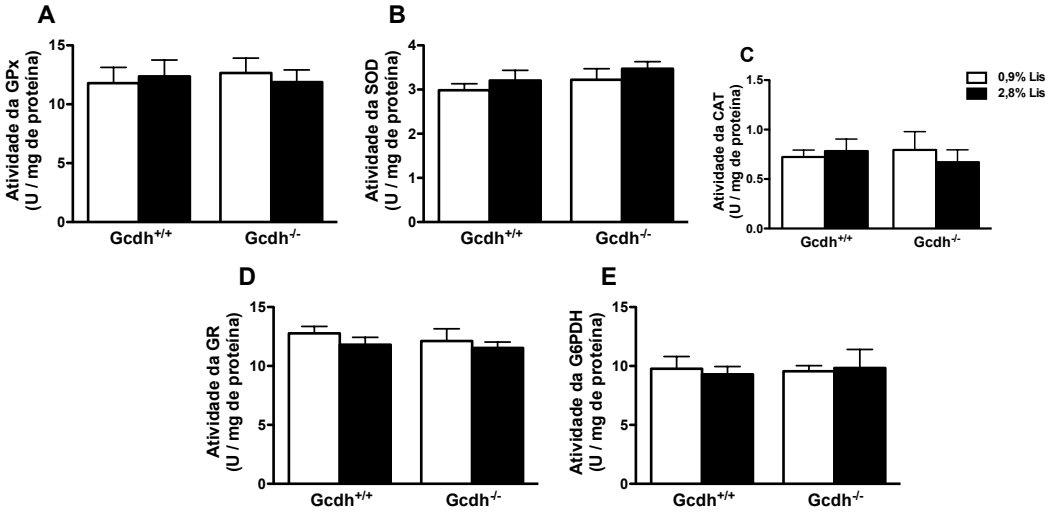


Figura 3.3.1 – Efeito da dieta crônica de lisina 2,8% sobre as atividades das enzimas glutationa peroxidase (GPx; A), superóxido dismutase (SOD; B), catalase (CAT; C), glutationa redutase (GR; D) e glicose-6-fosfato desidrogenase (G6PDH; E) em sobrenadante de córtex cerebral de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$. Os valores representam média ± desvio padrão da média (n=4-6). Os resultados foram analisados por teste *t* de Student para amostras independentes. Não houve diferença significativa entre os grupos.

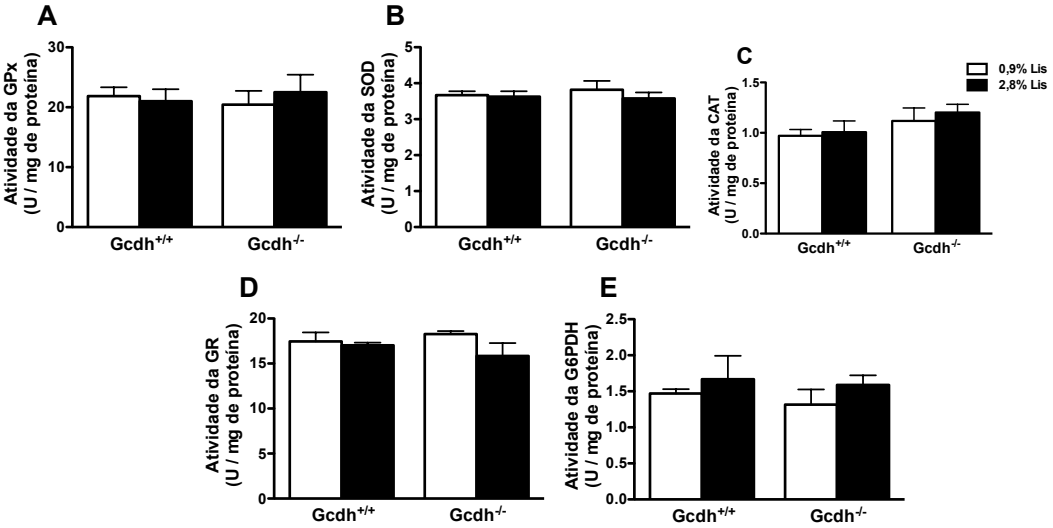
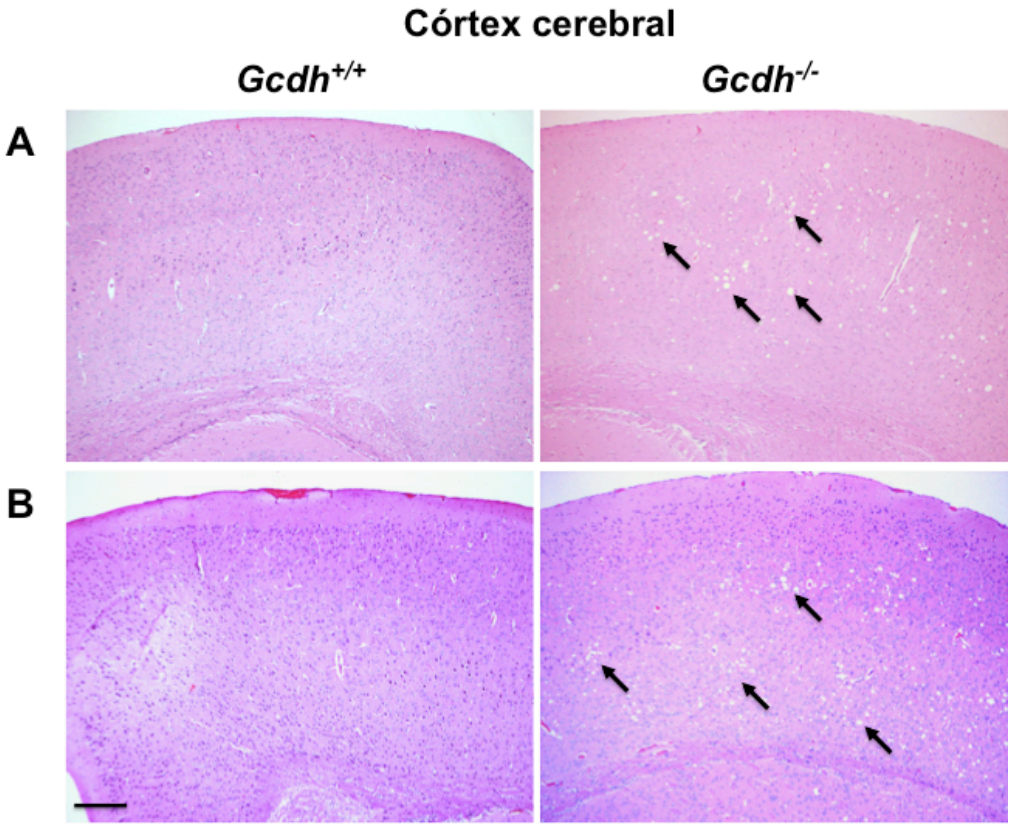


Figura 3.3.2 – Efeito da dieta crônica de lisina 2,8% sobre as atividades das enzimas glutationa peroxidase (GPx; A), superóxido dismutase (SOD; B), catalase (CAT; C), glutationa redutase (GR; D) e glicose-6-fosfato

desidrogenase (G6PDH; E) em sobrenadante de estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$. Os valores representam média ± desvio padrão da média (n=4-6). Os resultados foram analisados por teste *t* de Student para amostras independentes. Não houve diferença significativa entre os grupos.

3.4. Análise histológica do córtex cerebral e do estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ de 60 dias de vida submetidos a uma dieta crônica de lisina

A etapa seguinte deste trabalho foi investigar anormalidades histológicas através da coloração com HE em cérebro de camundongos *Gcdh*$^{-/-}$ submetidos a dieta normal (0,9% Lis) ou com 2,8% de Lis por 40 dias. Observamos a presença de uma intensa vacuolização no córtex cerebral, mas não no estriado de camundongos *Gcdh*$^{-/-}$ com 60 dias de vida submetidos a dieta normal (Figura 3.4, painel A). Além disso, os animais *Gcdh*$^{-/-}$ tratados com dieta rica em Lis apresentaram um grande número de vacúolos no estriado, sem que houvesse um aumento da vacuolização no córtex cerebral (Figura 3.4, painel B).


Córtex cerebral
Gcdh+/+
Gcdh-/-
A
B

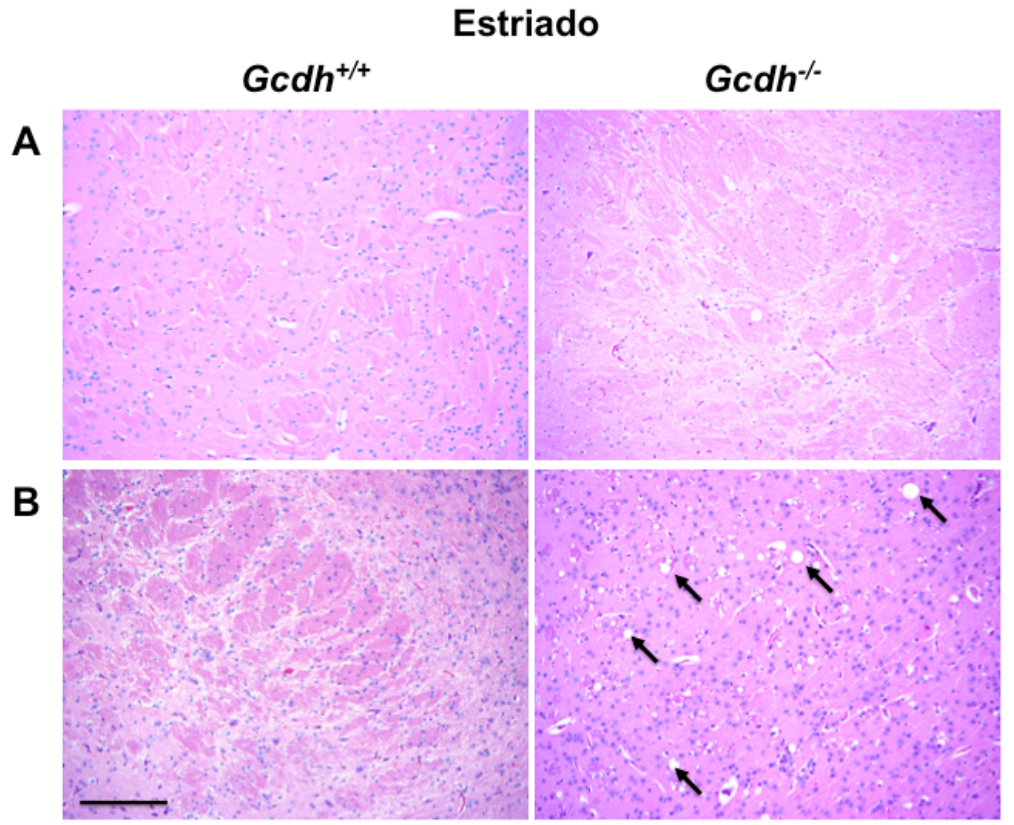

Estriado
Gcdh+/+
Gcdh-/-
A
B

Figura 3.5 – Coloração por Hematoxilina e Eosina do córtex cerebral e do estriado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ submetidos à dieta 0,9 % de Lis (A) ou 2,8% de Lis (B). As flechas indicam a presença de vacúolos. Imagens representativas foram obtidas de 3 animais. Aumento de ×200 (estriado) e ×100 (córtex cerebral).

PARTE III

Discussão e Conclusões

III.1. DISCUSSÃO

Pacientes acometidos pela AG I normalmente apresentam destruição estriatal aguda durante crises encefalopáticas associadas à descompensação metabólica, bem como leucoencefalopatia progressiva que afeta principalmente o córtex cerebral (Goodman e Frerman, 2001).

Apesar dos sintomas neurológicos e as anormalidades cerebrais ocorrerem na maioria dos indivíduos afetados não tratados, os mecanismos moleculares envolvidos no dano cerebral da doença ainda não estão bem esclarecidos. Além disso, praticamente todos os estudos sobre a patogênese desse distúrbio foram realizados em tecidos de animais selvagens com atividade normal da GCDH. Baseando-se nesses estudos, diversas hipóteses têm sido levantadas para explicar os danos neurológicos encontrados na doença, tais como a excitotoxicidade, alterações da bioenergética celular, comprometimento da integridade da BHE e do estado redox (Amaral *et al*., 2012a, 2012b; Ferreira *et al*., 2007; Figuera *et al*., 2006; Jafari *et al*., 2011; Kölker *et al*., 2002a, 2002b; Latini *et al*., 2005; Magni *et al*., 2012; Olivera *et al*., 2008; Rodrigues *et al*., 2013; Sauer *et al*., 2005; Ulrich *et al*., 1999; Zinnanti *et al*., 2006, 2007).

No que se refere à homeostase redox, alterações da mesma têm sido frequentemente relacionadas à morte celular, a doenças neurodegenerativas comuns, bem como à neurodegeneração presente em vários EIM, incluindo a AG I (Gu *et al*., 1998; Jafari *et al*., 2011; Lovell *et al*., 2000; Magni *et al*., 2009; Rodrigues *et al*., 2013). Estudos anteriores realizados *in vitro* ou *in vivo* em tecidos de ratos demonstraram que os ácidos orgânicos acumulados na AG I (AG e 3HG) induzem estresse oxidativo em culturas de células neurais e

em cérebro fresco de roedores (de Oliveira Marques *et al*., 2003; Jafari *et al*., 2011; Latini *et al*., 2002, 2005, 2007; Magni *et al*., 2009; Magni *et al*., 2012; Rodrigues *et al*., 2013).

O desenvolvimento de um modelo genético com atividade nula da GCDH (*Gcdh*$^{-/-}$) em camundongos, mais tarde aperfeiçoado com administração de dieta rica em Lis, tornou-se uma boa ferramenta para estudar a neuropatologia desse EIM, uma vez que reproduz o fenótipo bioquímico e neurológico dos pacientes, incluindo a leucoencefalopatia e a degeneração estriatal (Koeller *et al*., 2002: Zinnanti *et al*., 2006, 2007). Nesse particular, a suplementação de uma dieta rica em Lis (4,7 %) aos animais *Gcdh*$^{-/-}$ resultou em um aumento nas concentrações teciduais de AG e 3HG, além de provocar o aparecimento de lesões no estriado, semelhantes às que ocorrem em crianças afetadas pela AG I (Zinnanti et al., 2006).

A presente investigação foi elaborada usando cérebro, fígado e coração de camundongos jovens com idades entre 15 e 60 dias de vida com o objetivo primordial de avaliar a homeostase redox nesses animais em condições basais ou submetidos a uma sobrecarga aguda ou crônica de Lis. Animais selvagens e nocaute de 15 e 30 dias de vida foram submetidos a uma injeção aguda intraperitoneal de Lis (8 µmol/g), enquanto camundongos de 30 dias de idade receberam por 60 h uma dieta com 2,8 ou 4,7 % de Lis. Em outros experimentos, animais selvagens e nocaute receberam suplementação de 2,8 % desse aminoácido por 40 dias desde o 21º dia de vida. Nosso intuito foi o de mimetizar uma situação de descompensação metabólica com aumento dos metabólitos acumulados (AG e 3HG), induzida por sobrecarga aguda de Lis (injeção única i.p. ou dieta por um curto espaço

de tempo). Também objetivamos uma situação de sobrecarga crônica de Lis, com o intuito de estudar se altas concentrações desse aminoácido na dieta poderiam provocar alterações estruturais e morfológicas no cérebro dos animais selvagens e nocaute tratados.

Os seguintes parâmetros de estresse oxidativo foram avaliados: TBA-RS (dano oxidativo lipídico), conteúdo de grupamentos sulfidrilas e carbonilas (dano oxidativo proteico), oxidação de DCFH e nitratos e nitritos (espécies reativas), concentrações de GSH e atividades das enzimas antioxidantes GPx, SOD, CAT, GR e G6PDH (defesas antioxidantes). Os estudos histopatológicos foram realizados por técnicas de imunohistoquímica em córtex cerebral e estriado dos camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ de 30 dias de vida submetidos à dieta com alta concentração de Lis (4,7%) por 60 horas ou por técnicas histológicas em animais de 60 dias de vida tratados com uma dieta enriquecida com Lis (2,8%) por 40 dias, com o intuito de detectar alterações morfológicas e funcionais no SNC.

Primeiramente, os parâmetros foram avaliados as defesas antioxidantes, dano oxidativo proteico e oxidação de DCFH em cérebro total e fígado de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ de 15 dias de vida injetados intraperitonalmente com uma dose única de solução salina (controle) ou Lis (8 μmol/g). Em condições basais (injeção de salina) houve redução nas concentrações de GSH no fígado e do conteúdo de sulfidrilas no cérebro dos animais *Gcdh*$^{-/-}$, comparados aos *Gcdh*$^{+/+}$. A injeção de Lis por sua vez diminuiu ainda mais as concentrações de GSH no cérebro e o conteúdo de sulfidrilas no fígado, além de aumentar a formação de carbonilas e a oxidação de DCFH em ambos tecidos. Por fim, as atividades de todas as

enzimas antioxidantes diminuíram no cérebro, enquanto que as atividades da SOD e da CAT aumentaram no fígado dos camundongos *Gcdh*$^{-/-}$ que receberam Lis. Esses resultados evidenciam alterações do estado redox em camundongos *Gcdh*$^{-/-}$ no início de seu desenvolvimento em condições basais que se acentuam com a sobrecarga de Lis.

Investigamos também os parâmetros de estresse oxidativo em estruturas cerebrais (córtex cerebral, estriado e hipocampo) e tecidos periféricos (fígado e coração) de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ de 30 dias de vida. Não foram encontradas diferenças entre os dois grupos em condições basais (dieta normal) em todos os tecidos examinados, distintamente do que ocorreu com animais *Gcdh*$^{-/-}$ de 15 dias de idade. Em um segundo momento, investigamos os mesmos parâmetros de estresse oxidativo após uma única injeção i.p. de solução salina (NaCl 0,9 %) ou Lis (8 μmol/g) em animais de mesma idade. A injeção de Lis provocou um aumento das concentrações do AG e 3HG no cérebro de camundongos *Gcdh*$^{-/-}$, principalmente no estriado, mas também no córtex cerebral.

Verificamos também que a administração de Lis nos camundongos *Gcdh*$^{-/-}$ de 30 dias induziu peroxidação lipídica (aumento dos níveis de TBA-RS) em estriado e córtex cerebral, quando comparado aos animais *Gcdh*$^{+/+}$. A medida de TBA-RS indica principalmente a quantidade de malondialdeído (MDA) formado, produto da oxidação de ácidos graxos poliinsaturados de lipídios complexos. Por ser altamente tóxico, o MDA causa alterações nos resíduos de aminoácidos e mutações ao reagir com proteínas e bases do DNA, respectivamente (Halliwell e Gutteridge, 2007a). Além disso, o aumento significativo na oxidação de DCFH observado no estriado provavelmente

reflete uma produção aumentada de espécies reativas nesse tecido, que podem estar relacionados com os resultados mencionados anteriormente de concentrações mais elevadas de AG nessa estrutura cerebral. A medida direta de uma espécie reativa é de difícil realização, pois a alta reatividade da maioria dessas espécies (baixo tempo de meia-vida) não permite uma estabilidade adequada para sua medida. Contudo, a oxidação da sonda DCFH indica a quantidade de espécies reativas geradas no tecido. Dessa forma, os resultados obtidos na presente investigação sugerem um efeito pró-oxidante relevante da suplementação de Lis sobre lipídeos de membrana provavelmente mediado pela geração de ERO e ERN.

Uma diminuição das concentrações de GSH também foi observada no estriado e córtex cerebral dos animais *Gcdh*$^{-/-}$ de 30 dias de vida que receberam dose intraperiotoneal única de Lis, indicando um comprometimento das defesas antioxidantes não enzimáticas provavelmente como consequência do aumento na produção de radicais livres. A GSH é um efetivo sequestrador de radicais livres, protegendo grupamentos tióis, além de ser cofator da enzima GPx e da melatonina, e ajudando a reduzir as formas oxidadas de outros antioxidantes (do α-tocoferol e do ascorbato), o que atesta sua relevância como agente antioxidante. É possível, portanto, que o dano oxidativo demonstrado em nossa investigação nos camundongos *Gcdh*$^{-/-}$ seja em parte decorrente da diminuição do conteúdo de GSH no córtex cerebral e estriado de animais injetados com Lis.

A injeção de Lis também modificou as atividades das enzimas antioxidantes, aumentando as atividades das enzimas SOD e GR e diminuindo a atividade da GPx no estriado, enquanto que em córtex cerebral

apenas um aumento da atividade da SOD foi observado. Embora não tenhamos investigado os mecanismos que levaram ao aumento de algumas atividades enzimáticas, a indução da expressão da GR e da SOD a nível gênico pode ocorrer como um mecanismo compensatório em resposta ao aumento na formação de espécies reativas. Esses resultados podem ser devido ao fato de que ERO regulam a expressão de inúmeros genes, incluindo aqueles que codificam para enzimas antioxidantes por vias de sinalização específicas (Halliwell, 2006; Lakshminarayanan *et al*., 1998; Rushmore *et al*., 1991). Por outro lado, o aumento da atividade da SOD poderia também ser secundário ao aumento das concentrações do radical superóxido, convertendo-o ao peróxido de hidrogênio, enquanto o aumento da atividade da GR poderia facilitar a regeneração da GSH.

Já no hipocampo as alterações nos parâmetros de estresse oxidativo foram pouco evidentes e refletidas pelos aumentos moderados na oxidação de DCFH e da atividade da SOD. Em contraste aos achados cerebrais, não houve qualquer modificação dos parâmetros avaliados em fígado e coração dos animais *Gcdh*$^{-/-}$, indicando uma maior susceptibilidade do SNC ao dano oxidativo, principalmente do estriado, tecido no qual se encontraram concentrações mais elevadas de AG, quando comparado ao córtex cerebral.

Na etapa seguinte do trabalho investigamos os parâmetros de estresse oxidativo em córtex cerebral, estriado, hipocampo, fígado e coração de camundongos selvagens e nocautes para a enzima GCDH de 30 dias de vida submetidos à uma dieta normal (0,9% de Lis) ou a dietas enriquecidas com Lis (2,8% e 4,7%) por 60 horas. A suplementação de Lis na dieta levou a um aumento da peroxidação lipídica (níveis aumentados de MDA), da

produção de espécies reativas (aumento na oxidação de DCFH) e alterações das defesas antioxidantes (diminuição das concentrações de GSH e alterações das atividades das enzimas antioxidantes) em estriado e em córtex cerebral, mas não em hipocampo, fígado e coração, de animais *Gcdh*$^{-/-}$ comparados aos animais *Gcdh*$^{+/+}$ recebendo as mesmas dietas. Uma correlação inversa significativa entre as concentrações de GSH e a oxidação de DCFH também foi demonstrada, sugerindo que a diminuição das defesas antioxidantes cerebrais seja provavelmente secundária ao aumento na formação de espécies reativas. Dessa forma, podemos inferir que uma redução desse importante antioxidante cerebral possa reflitir seu alto consumo (oxidação) pelo aumento da produção de espécies reativas. Também não podemos excluir a possibilidade de que o dano oxidativo lipídico induzido pela dieta rica em Lis tenha ocorrido pela depleção de GSH.

Outro passo de nossa investigação foi o de comparar os níveis de TBA-RS, as concentrações de GSH e a atividade da enzima SOD em animais *Gcdh*$^{-/-}$ sintomáticos com assintomáticos após a ingestão da dieta enriquecida com Lis (4,7%). Verificamos que as alterações detectadas foram mais pronunciadas nos animais nocautes sintomáticos, sugerindo que o comprometimento da homeostase redox possa estar associado a efeitos deletérios da Lis e mais provavelmente dos ácidos orgânicos derivados da mesma no cérebro dos animais.

Finalmente, análises histopatológicas de córtex cerebral e estriado dos animais *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ de 30 dias de vida expostos a dieta com Lis 4,7% por 60 horas foram realizadas por técnicas de imunofluorescência. A marcação dos neurônios nos animais *Gcdh*$^{-/-}$ com NeuN não mostrou

diferenças significativas, tanto no número quanto na imunoreatividade, quando comparado ao grupo de camundongos selvagens (WT). Além disso, a marcação para Ki67 foi muito semelhante entre os grupos de animais *Gcdh*$^{-/-}$ e WT, descartando dano neuronal significativo.

No que diz respeito às células gliais, não foram observadas diferenças no número de células S100b e GFAP positivas em camundongos *Gcdh*$^{-/-}$ expostos à dieta com alta concentração de Lis (4,7%) em relação aos animais selvagens, o que afasta a possibilidade de astrogliose. No entanto, detectamos um aumento moderado na imunoreatividade para GFAP e um inchamento das células marcadas com S100b em comparação com as dos camundongos selvagens, indicando estresse astrocitário. Por outro lado, verificamos um número aumentado de células positivas para nitrotirosina (YNO2) e um aumento expressivo da marcação para a enzima óxido nítrico sintetase induzível (iNOS), quando comparado com o grupo de animais WT. Esses dados histopatológicos indicam um aumento na expressão de marcadores de estresse oxidativo induzido por ERN.

Tomados em seu conjunto, os presentes resultados indicam uma disfunção da homeostase redox em córtex cerebral e estriado de animais *Gcdh*$^{-/-}$ de 15 e 30 dias de vida expostos a uma sobrecarga de Lis.

No que se refere a animais de 60 dias de vida, não houve nenhuma alteração dos parâmetros de estresse oxidativo avaliados no córtex cerebral e estriado dos camundongos *Gcdh*$^{-/-}$ submetidos à dieta normal, embora os níveis de TBA-RS tenham aumentado moderadamente nestas estruturas cerebrais após dieta enriquecida com Lis (2,8%) por 40 dias a partir do 21º dia de idade. Por outro lado, essa dieta crônica não alterou os outros

parâmetros de estresse oxidativo avaliados (concentrações de GSH e atividade das enzimas antioxidantes GPx, SOD, CAT, GR e G6PDH). Infelizmente não pudemos administrar 4,7 % de Lis a esses animais, pois a maioria deles tem um desenlace fatal após 5 dias de tratamento.

Com relação à avaliação histológica das estruturas cerebrais nos animais *Gcdh*$^{-/-}$ de 60 dias tratados com dieta normal (0,9 % de Lis), realizada através da coloração por HE, observamos uma intensa vacuolização no córtex cerebral de camundongos *Gcdh*$^{-/-}$ sem alterações no estriado. Além disso, quando submetemos os camundongos *Gcdh*$^{-/-}$ a dieta rica em Lis (2,8%) por 40 dias desde o 21º dia de vida, verificamos a presença de um grande número de vacúolos no estriado desses animais, enquanto que a vacuolização cortical não foi aumentada. Nossos dados indicam que o estriado dos camundongos *Gcdh*$^{-/-}$ pode estar sendo progressivamente lesado pelo aumento persistente da Lis e mais provavelmente dos metabólitos acumulados na AG I derivados da Lis, particularmente o AG. Neste sentido, um estudo recente observou uma degeneração estriatal tardia em ratos tratados com AG no primeiro dia de vida (Olivera-Bravo *et al*., 2011).

Um estudo anterior demonstrou pequena perda neuronal e ativação astrocitária em córtex cerebral e estriado de camundongos *Gcdh*$^{-/-}$ adultos tratados com uma sobrecarga de Lis na dieta por 45 dias (Zinnanti *et al*., 2006), indicando que a Lis e/ou seus derivados ácidos sejam deletérios para o SNC. Tomados em seu conjunto este estudo e os resultados de nossa presente investigação estão de acordo com estudos neuroradiológicos feitos em pacientes com AG I, demonstrando que, além das alterações do córtex

cerebral, uma lesão estriatal crônica também acomete pacientes com essa doença (Neumaier-Probst *et al*., 2004).

Assim, a avaliação da homeostase redox ao longo do desenvolvimento de camundongos selvagens e *Gcdh*$^{-/-}$, nas idades de 15, 30 e 60 dias de vida, mostrou importantes diferenças nas respostas do organismo frente à uma sobrecarga aguda ou crônica de Lis (através da administração i.p. de Lis ou dieta enriquecida com esse aminoácido). As alterações mais significativas dos parâmetros de estresse oxidativo ocorreram nos animais mais jovens deficientes em GCDH com 15 e 30 dias de vida, enquanto que nos animais *Gcdh*$^{-/-}$ com 60 dias de vida praticamente nenhuma alteração da homeostase redox foi observada, mesmo após a suplementação da dieta com 2,8% de Lis por um longo período. Já os animais nocaute mais jovens, de 15 dias de vida, em dieta normal sem a presença de altas concentrações de Lis, mostraram importantes alterações nas defesas antioxidantes, tendo níveis diminuídos de GSH no cérebro e menor quantidade de grupamentos sulfidrilas no fígado, quando comparados aos animais WT. Após a injeção de Lis, outros parâmetros se alteraram, indicando aumento na produção de ERO e dano proteico. Neste particular, é possível que a imaturidade da BHE nesse período do desenvolvimento explique as alterações do estado redox observadas em animais dessa idade após a administração aguda de uma alta dose de Lis que leva à produção aumentada de AG no cérebro (Zinnati *et al*., 2006, 2014). Assim, acreditamos que os efeitos encontrados estejam sendo mediados pelo presença do AG, que já foi apontado como agente causador de efeitos deletérios em outros estudos realizados em cérebro de roedores (Latini *et al*., 2005: Magni *et al*., 2009, Rodrigues *et al*., 2013).

Na idade de 30 dias, a indução de estresse oxidativo no córtex cerebral e especialmente no estriado dos camundongos *Gcdh*$^{-/-}$ somente foi detectada após a sobrecarga de Lis, através de uma única injeção única ou pela dieta de curta duração enriquecida neste aminoácido, não sendo observada com a injeção de solução salina ou com dieta normal. Como já evidenciado em outros trabalhos, o aumento nas concentrações intracerebrais de AG parece estar relacionada com a sua produção pelas células neurais e pela dificuldade desse ácido dicarboxílico de atravessar as membranas cerebrais, levando ao seu acúmulo no SNC (Hoffmann *et al*., 1993, 1994; Jafari *et al*., 2011; Sauer *et al*., 2006; Stellmer *et al*., 2007; Zinnanti *et al*., 2007). Assim, acreditamos que a Lis após entrar no cérebro pela BHE seja convertida principalmente em AG, cujas concentrações aumentam pela impossibilidade de metabolizá-lo pela falta da enzima GCDH e pela dificuldade do mesmo em sair do cérebro (Jafari *et al*., 2011; Zinnanti *et al*., 2007).

Analisando ainda as diferenças dos resultados encontrados em córtex cerebral e estriado, nessa última estrutura se observaram efeitos mais significativos do que no córtex cerebral. A vulnerabilidade do estriado ao estresse oxidativo em comparação com córtex cerebral, e ainda hipocampo e os tecidos periféricos coração e fígado, pode ser devida às concentrações maiores dos metabólitos derivados da Lis (AG e 3HG) ou a uma suscetibilidade maior do estriado e em menor intensidade do córtex cerebral a esses compostos. Neste particular, é de se notar que tanto na condição humana quanto no modelo genético aqui utilizado essas regiões cerebrais são mais afetadas em relação a outras.

Resultados experimentais anteriores demonstraram que os ácidos AG e 3HG são pró-oxidantes e podem reduzir as defesas antioxidantes no SNC (de Oliveira Marques *et al*., 2003; Jafari *et al*., 2011; Latini *et al*., 2002, 2005, 2007; Magni *et al*., 2009; Magni *et al*., 2012; Rodrigues *et al*., 2013), desempenhando possivelmente um importante papel através desse mecanismo no dano cerebral característico da AG I. Não se pode porém afastar a possibilidade que a própria Lis seja em parte responsável pelos efeitos encontrados em nosso trabalho, uma vez que foi previamente demonstrado que esse aminoácido induz estresse oxidativo *in vitro* e *in vivo* no cérebro de ratos jovens (Seminotti *et al*., 2008; Seminotti *et al*., 2011) e que não se conhece as concentrações intracerebrais da Lis nos pacientes afetados por essa doença. Nesse particular, a restrição de Lis na dieta tem sido usada com sucesso para prevenir a degeneração estriatal em pacientes com AG I e mais recentemente a suplementação dietética com arginina ou homoarginina tem sido demonstrada ser benéfica para os animais *Gcdh*$^{-/-}$ e seres humanos afetados por esse distúrbio provavelmente por competirem com a Lis pelo transportador na BHE (Jafari *et al*., 2011; Kölker *et al*., 2012; Strauss *et al*., 2013; Strauss *et al*., 2011; Strauss e Morton, 2003; Zinnanti *et al*., 2007).

Enfatize-se ainda que o estresse oxidativo é uma condição deletéria à célula (Halliwell e Gutteridge, 2007a) e principalmente ao SNC que possui poucas defesas antioxidantes, quando comparado a 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 (Behl e Moosmann, 2002; Berg e Youdim, 2006; Bogdanov *et al*., 2001; Halliwell e Gutteridge, 1996; Karelson *et al*., 2001; Mancuso *et al*., 2006; Méndez-Álvarez *et al*., 2001; Perez-Severiano *et al*., 2000; Stoy *et al*., 2005).

Concluindo, relatamos pela primeira vez que uma sobrecarga de Lis aguda induz estresse oxidativo principalmente em tecidos cerebrais, mas também em fígado, de camundongos nocaute para a enzima GCDH, especialmente nos estágios iniciais do desenvolvimento (15 e 30 dias de vida). Já que uma produção elevada de espécies reativas, as quais induzem dano a componentes celulares fundamentais para o funcionamento normal dos tecidos (proteínas, lipídios e DNA), seria potencialmente deletéria ao SNC, o uso de agentes antioxidantes poderia representar uma nova abordagem terapêutica, junto com outras medidas, no tratamento dos pacientes afetados por esse distúrbio genético.

III.2. CONCLUSÕES

Nossos resultados indicam uma alteração da homeostase redox celular em animais deficientes da enzima GCDH (modelo nocaute de AG I; *Gcdh*$^{-/-}$), principalmente nas estruturas cerebrais estriado e córtex cerebral, em estágios iniciais do desenvolvimento que se acentua quando esses animais recebem uma injeção única de altas concentrações de Lis ou dieta de curta duração enriquecida em Lis.

As alterações dos parâmetros de estresse oxidativo foram mais evidentes no estriado em relação ao córtex cerebral.

Também foram detectadas vacuolizações no córtex cerebral de animais *Gcdh*$^{-/-}$ com 60 dias de vida, processo que não se acentuou por uma dieta crônica com alta concentração de Lis. No entanto, o estriado dos animais com 60 dias de vida em dieta normal não apresentou as mesmas alterações histológicas, que apenas ocorreram quando da dieta crônica com Lis por 40 dias.

III.3. PERPECTIVAS

Estudar o efeito de uma sobrecarga aguda de Lis (injeção i.p.) sobre a homeostase redox e energética em animais mais jovens (5, 7 e 10 dias de vida).

Estudar parâmetros de homeostase redox em filhotes *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ (cérebro, fígado e coração) de mães homozigotas submetidas a uma sobrecarga crônica de Lis (dieta), bem como sobre o desenvolvimento neuromotor e o comportamento (aprendizado/memória) da prole em idade adulta.

Avaliar os tecidos cerebrais de camundongos *Gcdh*$^{+/+}$ e *Gcdh*$^{-/-}$ com 7, 15 e 30 dias de vida submetidos a uma sobrecarga de Lis por técnicas histológicas e por imunohistoquímica, bem como por “imunoblotting”.

Avaliar a homeostase redox em tecidos (sangue, fígado e músculo esquelético) de pacientes com AG I.

REFERÊNCIAS

Aebi, H. Catalase in vitro. Methods Enzymol 105: 121-1261, 1984.

Amaral, A.U., Seminotti, B., Cecatto, C., Fernandes, C.G., Busanello, E.N., Zanatta, Â., Kist, L.W., Bogo, M.R., de Souza, D.O.G., Woontner, M., Goodman, S., Koeller, D.M., Wajner, M. Reduction of Na+,K+-ATPase activity and expression. In cerebral córtex of glutaryl-CoA dehydrogenase deficiente mice: a possible mechanism for brain injury in glutaric aciduria type I. Mol Genet Metab 107: 375-382, 2012a.

Amaral, A.U., Cecatto, C., Seminotti, B., Zanatta, Â., Fernandes, C.G., Busanello, E.N., Braga, L.M., Ribeiro, C.A., de Souza, D.O.G., Woontner, M., Koeller, D.M., Goodman, S., Wajner, M. Marked reduction of Na+,K+-ATPase and creatine kinase activities induced by acute lysine administration in glutaryl-CoA dehydrogenase deficient mice. Mol Genet Metab 107: 81-86, 2012b.

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

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

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

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

Bergman, I., Finegold, D., Gartner, J.C., Zitelli, B.J., Claassen, D., Scarano, J., Roe, C.R., Stanley, C., Goodman, S.I. Acute profound dystonia in infants with glutaric acidemia. Pediatrics 83: 228-34, 1989.

Bjugstad, K.B., Zawada, W.M., Goodman, S., Freed, C.R. IGF-1 and bFGF reduce glutaric acid and 3-hydroxyglutaric acid toxicity in striatal cultures. J Inherit Metab Dis 24: 631-647, 2001.

Bodamer, O.A., Gruber, S., Stockler-Ipsiroglu, S. Nuclear magnetic resonance spectroscopy in glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 27: 877-883, 2004.

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.

Boveris, A., Chance, B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 134: 707-716, 1973.

Boy, N., Haege, G., Heringer, J., Assmann, B., Mühlhausen, C., Ensenauer, R., Maier, E.M., Lücke, T., Hoffmann, G.F., Müller, E., Burgard, P., Kölker, S. Low lysine diet in glutaric aciduria type I - effect on anthropometric and biochemical follow-up parameters. J Inherit Metab Dis 36: 525-33, 2013.

Brismar, J., Ozand, P.T. CT and MR of the brain in glutaric acidemia type I: a review of 59 published cases and a report of 5 new patients. AJNR Am J Neuroradiol 16: 675-68, 1995.

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

Burlina, A,P,, Zara, G., Hoffmann, G.F., Zschocke, J., Burlina, A.B. Management of movement disorders in glutaryl-CoA dehydrogenase

deficiency: anticholinergic drugs and botulinum toxin as additional therapeutic options. J Inherit Metab Dis 27: 911-5, 2004.

Busanello, E.N., Pettenuzzo, L., Botton, P.H., Pandolfo, P., de Souza D.O., Woontner, M., Goodman, S., Koeller, D., Wajner, M. Neurodevelopmental and cognitive behavior of glutaryl-CoA dehydrogenase deficient knockout mice. Life Sci 92: 137-142, 2013.

Busquets, C., Soriano, M., de Almeida, I.T., Garavaglia, B., Rimoldi, M., Rivera, I., Uziel, G., Cabral, A., Coll, M.J., Ribes, A. Mutation analysis of the GCDH gene in Italian and Portuguese patients with glutaric aciduria type I. Mol Genet Metab 71: 535-537, 2000.

Calberg I., Mannervik B., Glutathione Reductase. Method Enzymol 113: 484-90, 1985.

Campistol, J., Ribes, A., Alvarez, L., Christensen, E., Millington, D.S. Glutaric aciduria type I: unusual biochemical presentation. J Pediatr 121: 83-86, 1992.

Carman, K.B., Aydogdu, S.D., Yakut, A., Yarar, C. Glutaric aciduria type 1 presenting as subdural haematoma. J Paediatr Child Health 48: 712, 2012.

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. Chapman & Hall, London, 1982.

Couce, M.L., López-Suárez, O., Bóveda, M.D., Castiñeiras, D.E., Cocho, J.A., García-Villoria, J., Castro-Gago, M., Fraga, J.M., Ribes, A. Glutaric aciduria type I: outcome of patients with early- versus late-diagnosis. Eur J Paediatr Neurol 17: 383-389, 2013.

Dalcin, K.B., Rosa, R.B., Schmidt, A.L., Winter, J.S., Leipnitz, G., Dutra-Filho, C.S., Wannmacher, C.M.D., Porciúncula, L.O., Souza, D.O., Wajner, M. Age and brain structural related effects of glutaric and 3-hydroxyglutaric acids on glutamate binding to plasma membranes during rat brain development. Cell Mol Neurobiol 27: 805-818, 2007.

Danbolt, N.C. Glutamate uptake. Prog Neurobiol 65: 1-105, 2001.

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

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

Drigo, P., Piovan, S., Battistella, P.A., Della Puppa, A., Burlina, A.B. Macrocephaly, subarachnoid fluid collection, and glutaric aciduria type I. J Child Neurol 11: 414-417, 1996.

Ferreira, G.C., Viegas, C.M., Schuck, P.F., Tonin, A., Ribeiro, C.A., Coelho Dde, M., Dalla-Costa, T., Latini, A., Wyse, A.T., Wannmacher, C.M., Vargas, C.R., Wajner, M. Glutaric acid administration impairs energy metabolism in midbrain and skeletal muscle of young rats. Neurochem Res 30: 1123-1131, 2005.

Ferreira, G.C., Tonin, A., Schuck, P.F., Viegas, C.M., Ceolato, P.C., Latini, A., Perry, M.L., Wyse, A.T., Dutra-Filho, C.S., Wannmacher, C.M., Vargas, C.R., Wajner, M. Evidence for a synergistic action of glutaric and 3-hydroxyglutaric acids disturbing rat brain energy metabolism. Int J Dev Neurosci 25: 391-398, 2007.

Ferreira, G.C., Schuck, P.F., Viegas, C.M., Tonin, A., Latini, A., Dutra-Filho, C.S., Wyse, A.T., Wannmacher, C.M., Vargas, C.R., Wajner, M. Energy

metabolism is compromised in skeletal muscle of rats chronically-treated with glutaric acid. Metab. Brain Dis 22: 111-123, 2007.

Fighera, M.R., Royes, L.F., Furian, A.F., Oliveira, M.S., Fiorenza, N.G., Frussa-Filho, R., Petry, J.C., Coelho, R.C., Mello, C.F. GM1 ganglioside prevents seizures, Na+,K+-ATPase activity inhibition and oxidative stress induced by glutaric acid and pentylenetetrazole. Neurobiol Dis 22: 611-623, 2006.

Flott-Rahmel, B., Falter, C., Schluff, P., Fingerhut, R., Christensen, E., Jakobs, C., Musshoff, U., Fautek, J.D., Deufel, T., Ludolph, A., Ullrich, K. Nerve cell lesions caused by 3-hydroxyglutaric acid: a possible mechanism for neurodegeneration in glutaric acidaemia I. J Inherit Metab Dis 20: 387-390, 1997.

Freudenberg, F., Lukacs, Z., Ullrich, K. 3-Hydroxyglutaric acid fails to affect the viability of primary neuronal rat cells. Neurobiol Dis 16: 581-584, 2004

Gokmen-Ozel, H., MacDonald, A., Daly, A., Ashmore, C, Preece MA, Hendriksz C, Vijay S, Chakrapani A. Dietary practices in glutaric aciduria type 1 over 16 years. J Hum Nutr Diet 25: 514-9, 2012.

Goodman, S.I., Markey, S.P., Moe, P.G., Miles, B.S., Teng, C.C. Glutaric aciduria; a "new" disorder of amino acid metabolism. Biochem Med 12: 12-21, 1975.

Goodman, S.I., Norenberg, M.D., Shikes, R.H., Breslich, D.J., Moe, P.G. Glutaric aciduria: biochemical and morphologic considerations. J Pediatr 90: 746-750, 1977.

Goodman, S.I., Stein, D.E., Schlesinger, S., Christensen, E., Schwartz, M., Greenberg, C.R., Elpeleg, O.N. Glutaryl-CoA dehydrogenase mutations in glutaric acidemia (type I): review and report of thirty novel mutations. Hum Mutat 12: 141-144, 1998.

Goodman, S.I., Frerman, F. Organic acidemias due to defects in lysine oxidation: 2-ketoadipic academia and glutaric acidemia, 8 ed. McGraw-Hill, New York, 2001.

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.

Halliwell, B. Free radicals, proteins and DNA: oxidative damage versus redox regulation. Biochem Soc Trans. 24: 1023-1027, 1996.

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

Halliwell, B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141: 312-322, 2006.

Halliwell, B., Gutteridge, J.M.C. Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Oxford University Press Inc., Oxford, pp. 187–340, 2007.

Harting, I., Neumaier-Probst, E., Seitz, A., Maier, E.M., Assmann, B., Baric, I., Troncoso, M., Muhlhausen, C., Zschocke, J., Boy, N.P., Hoffmann, G.F., Garbade, S.F., Kolker, S. Dynamic changes of striatal and extrastriatal abnormalities in glutaric aciduria type I. Brain 132: 1764-1782, 2009.

Hedlund, G.L., Longo, N., Pasquli, M. Glutaric acidemia type 1. Am J Med Genet C Semin Med Genet 142C: 86-94, 2006.

Heyes, M.P. Hypothesis: a role for quinolinic acid in the neuropathology of glutaric aciduria type I. Can J Neurol Sci 14: 441-443, 1987.

Hoffmann, G.F., Trefz, F.K., Barth, P.G., Bohles, H.J., Biggemann, B., Bremer, H.J., Christensen, E., Frosch, M., Hanefeld, F., Hunneman, D.H. et al. Glutaryl-coenzyme A dehydrogenase deficiency: a distinct encephalopathy. Pediatrics 88: 1194-1203, 1991.

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. Selective screening for inborn errors of metabolism--past, present and future. Eur J Pediatr 153: S2-8, 1994.

Hoffmann, G.F., Athanassopoulos, S., Burlina, A.B., Duran, M., de Klerk, J.B., Lehnert, W., Leonard, J.V., Monavari, A.A., Muller, E., Muntau, A.C., Naughten, E.R., Plecko-Starting, B., Superti-Furga, A., Zschocke, J., Christensen, E. Clinical course, early diagnosis, treatment, and prevention of disease in glutaryl-CoA dehydrogenase deficiency. Neuropediatrics 27: 115-123, 1996.

Hoffmann, G.F., Zschocke, J. Glutaric aciduria type I: from clinical, biochemical and molecular diversity to successful therapy. J Inherit Metab Dis 22: 381-391, 1999.

Hoffmann, G.F., von Kries, R., Klose, D., Lindner, M., Schulze, A., Muntau, A.C., Roschinger, 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.

Jafari, P., Braissant, O., Bonafé, L., Ballhausen, D. The unsolved puzzle of neuropathogenesis in glutaric aciduria type I. Mol Genet Metab 104: 425-437, 2011.

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.

Koeller, D.M., Woontner, M., Crnic, L.S., Kleinschmidt-DeMasters, B., Stephens, J., Hunt, E.L., Goodman, S.I. Biochemical, pathologic and behavioral analysis of a mouse model of glutaric acidemia type I. Hum Mol Genet 11: 347-357, 2002.

Kölker, S., Kohr, G., Ahlemeyer, B., Okun, J.G., Pawlak, V., Horster, F., Mayatepek, E., Krieglstein, J., Hoffmann, G.F. Ca(2+) and Na(+) dependence of 3-hydroxyglutarate-induced excitotoxicity in primary neuronal cultures from chick embryo telencephalons. Pediatr Res 52: 199-206, 2002a.

Kölker, S., Okun, J.G., Ahlemeyer, B., Wyse, A.T., Horster, F., Wajner, M., Kohlmuller, D., Mayatepek, E., Krieglstein, J., Hoffmann, G.F. Chronic treatment with glutaric acid induces partial tolerance to excitotoxicity in neuronal cultures from chick embryo telencephalons. J Neurosci Res 68: 424-431, 2002b

Kölker, S., Koeller, D.M., Sauer, S., Horster, F., Schwab, M.A., Hoffmann, G.F., Ullrich, K., Okun, J.G. Excitotoxicity and bioenergetics in glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 27: 805-812, 2004.

Kölker, S., Garbade, S.F., Greenberg, C.R., Leonard, J.V., Saudubray, J.M., Ribes, A., Kalkanoglu, H.S., Lund, A.M., Merinero, B., Wajner, M., Troncoso, M., Williams, M., Walter, J.H., Campistol, J., Marti-Herrero, M., Caswill, M., Burlina, A.B., Lagler, F., Maier, E.M., Schwahn, B., Tokatli, A., Dursun, A., Coskun, T., Chalmers, R.A., Koeller, D.M., Zschocke, J., Christensen, E., Burgard, P., Hoffmann, G.F. Natural history, outcome, and treatment efficacy in children and adults with glutaryl-CoA dehydrogenase deficiency. Pediatr Res 59: 840-847, 2006.

Kölker, S., Garbade, S.F., Boy, N., Maier, E.M., Meissner, T., Mühlhausen, C., Hennermann, J.B., Lücke, T., Häberle, J., Baumkötter, J., Haller, W., Muller, E., Zschocke, J., Burgard, P., Hoffmann, G.F. Decline of acute encephalopathic crises in children with glutaryl-CoA dehydrogenase deficiency identified by newborn screening in Germany. Pediatr Res 62: 357-363, 2007.

Kölker, S., Sauer, S.W., Hoffmann, G.F., Müller, I., Morath, M.A., Okun, J.G. Pathogenesis of CNS involvement in disorders of amino and organic acid metabolism. J Inherit Metab Dis 31: 194-204, 2008.

Kölker, S., Boy, S.P., Heringer, J., Muller, E., Maier, E.M., Ensenauer, R., Muhlhausen, C., Schlune, A., Greenberg, C.R., Koeller, D.M., Hoffmann, G.F., Haege, G., Burgard, P. Complementary dietary treatment using lysine-free, arginine-fortified amino acid supplements in glutaric aciduria type I - A decade of experience. Mol Genet Metab 107: 72-80, 2012.

Lakshminarayanan, V., Drab-Weiss, E.A., Roebuck, K.A. H_2O_2 and tumor necrosis factor-alpha induce differential binding of the redox-responsive transcription factors AP-1 and NF-kappaB to the interleukin-8 promoter in endothelial and epithelial cells. J Biol Chem 273: 32670–32678, 1998.

Latini, A., Borba Rosa, R., Scussiato, K., Llesuy, S., Bello-Klein, A., Wajner, M. 3-Hydroxyglutaric acid induces oxidative stress and decreases the antioxidant defenses in cerebral cortex of young rats. Brain Res 956: 367-373, 2002.

Latini, A., Rodriguez, M., Borba Rosa, R., Scussiato, K., Leipnitz, G., Reis de Assis, D., da Costa Ferreira, G., Funchal, C., Jacques-Silva, M.C., Buzin, L., Giugliani, R., Cassina, A., Radi, R., Wajner, M. 3-Hydroxyglutaric acid moderately impairs energy metabolism in brain of young rats. Neuroscience 135: 111-120, 2005a.

Latini, A., Scussiato, K., Leipnitz, G., Dutra-Filho, C.S., Wajner, M. Promotion of oxidative stress by 3-hydroxyglutaric acid in rat striatum. J Inherit Metab Dis 28: 57-67, 2005b.

Latini, A., Ferreira, G.C., Scussiato, K., Schuck, P.F., Solano, A.F., Dutra-Filho, C.S., Vargas, C.R., Wajner, M. Induction of oxidative stress by chronic and acute glutaric acid administration to rats. Cell Mol Neurobiol 27: 423-438, 2007.

Lee, C.S., Chien, Y.H., Peng, S.F., Cheng, P.W., Chang, L.M., Huang, A.C., Hwu, W.L., Lee, N.C. Promising outcomes in glutaric aciduria type I patients detected by newborn screening. Metab Brain Dis 28: 61-7, 2013.

Lehnert, W., Sass, J.O. Glutaconyl-CoA is the main toxic agent in glutaryl-CoA dehydrogenase deficiency (glutaric aciduria type I). Med Hypotheses 65: 330-333, 2005.

Leibel, R.L., Shih, V.E., Goodaman, S., Bauman, M.L., McCabe, E.R., Zwerdling, R.G., Bergman, I., Costello, C. Glutaric acidemia: a metabolic disorder causing progressive choreoathetosis. Neurology 30: 1163-168, 1980.

Lindner, M., Ho, S., Fang-Hoffmann J, Hoffmann GF, Kölker S. Neonatal screening for glutaric aciduria type I: strategies to proceed. J Inherit Metab Dis 29: 378-82, 2006.

Lovell, M.A., Xie, C., Gabbita, S.P., Markesbery, W.R. Decreased thioredoxin and increased thioredoxin reductase levels in Alzheimer's disease brain. Free Radic Biol Med 28: 418-427, 2000.

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

Lund, T.M., Christensen, E., Kristensen, A.S., Schousboe, A., Lund, A.M. On theneurotoxicity of glutaric, 3-hydroxyglutaric, and trans-glutaconic acids in glutaric acidemia type I. J Neurosci Res 77: 143-147, 2004.

Magni, D.V., Furian, A.F., Oliveira, M.S., Souza, M.A., Lunardi, F., Ferreira, J., Mello, C.F., Royes, L.F., Fighera, M.R. Kinetic characterization of l-[(3)H]glutamate uptake inhibition and increase oxidative damage induced by glutaric acid in striatal synaptosomes of rats. Int J Dev Neurosci 27:65-72, 2009.

Magni, D.V., Brüning, C.A., Gai, B.M., Quines, C.B., Rosa, S.G., Fighera, M.R., Nogueira, C.W. m-Trifluoromethyl diphenyl diselenide attenuates glutaric acid-induced seizures and oxidative stress in rat pups: involvement of the γ-aminobutyric acidergic system. J Neurosci Res 90: 1723-1731, 2012.

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

Marklund, S.L. Pyrogallol autoxidation. In Handbook for oxygen radical research, Boca Raton, FL: CRC Press, pp 243–247, 1985.

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

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.

Merinero, B., Perez-Cerda, C., Font, L.M., Garcia, M.J., Aparicio, M., Lorenzo, G., Martinez Pardo, M., Garzo, C., Martinez-Bermejo, A., Pascual Castroviejo,

I. Variable clinical and biochemical presentation of seven Spanish cases with glutaryl-CoA-dehydrogenase deficiency. Neuropediatrics 26: 238-242, 1995.

Mühlhausen, C., Ott, N., Chalajour, F., Tilki, D., Freudenberg, F., Shahhossini, M., Thiem, J., Ullrich, K., Braulke, T., Ergün, S. Endothelial effects of 3-hydroxyglutaric acid: implications for glutaric aciduria type I. Pediatr Res 59: 196-202, 2006.

Neumaier-Probst, E., Harting, I., Seitz, A., Ding, C., Kölker, S. Neuroradiological findings in glutaric aciduria type I (glutaryl-CoA dehydrogenase deficiency). J Inherit Metab Dis 27: 869-76, 2004.

Nunes, J., Loureiro, S., Carvalho, S., Pais, R.P., Alfaiate, C., Faria, A., Garcia, P., Diogo, L. Brain MRI findings as an important diagnostic clue in glutaric aciduria type 1. Neuroradiol J 26: 155-161, 2013.

Olivera-Bravo, S., Fernandez, A., Sarlabos, M.N., Rosillo, J.C., Casanova, G., Jimenez, M., Barbeito, L. Neonatal astrocyte damage is sufficient to trigger progressive striatal degeneration in a rat model of glutaric acidemia-I. PLoS ONE 6: e20831, 2011.

Olivera-Bravo, S., Isasi, E., Fernández, A., Rosillo, J.C., Jiménez, M., Casanova, G., Sarlabós, M.N., Barbeito, L. White Matter Injury Induced by Perinatal Exposure to Glutaric Acid. Neurotox Res [Epub ahead of print], 2013.

Olivera, S., Fernandez, A., Latini, A., Rosillo, J.C., Casanova, G., Wajner, M., Cassina, P., Barbeito, L. Astrocytic proliferation and mitochondrial dysfunction induced by accumulated glutaric acidemia I (GAI) metabolites: possible implications for GAI pathogenesis. Neurobiol Dis 32: 528-534, 2008.

Perez-Duenas, B., De La Osa, A., Capdevila, A., Navarro-Sastre, A., Leist, A., Ribes, A., Garcia-Cazorla, A., Serrano, M., Pineda, M., Campistol, J. Brain

injury in glutaric aciduria type I: the value of functional techniques in magnetic resonance imaging. Eur J Paediatr Neurol 13: 534-540, 2009.

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.

Pfeil, J., Listl, S., Hoffmann, G.F., Kölker, S., Lindner, M., Burgard, P. Newborn screening by tandem mass spectrometry for glutaric aciduria type 1: a cost-effectiveness analysis. Orphanet J Rare Dis 8:167, 2013.

Pineda, M., Ribes, A., Busquets, C., Vilaseca, M.A., Aracil, A., Christensen, E. Glutaric aciduria type I with high residual glutaryl-CoA dehydrogenase activity. Dev Med Child Neurol 40: 840-2, 1998.

Porciuncula, L.O., Dal-Pizzol, A., Jr., Coitinho, A.S., Emanuelli, T., Souza, D.O., Wajner, M. Inhibition of synaptosomal [^{3}H]glutamate uptake and [^{3}H]glutamate binding to plasma membranes from brain of young rats by glutaric acid in vitro. J Neurol Sci 173: 93-96, 2000.

Porciuncula, L.O., Emanuelli, T., Tavares, R.G., Schwarzbold, C., Frizzo, M.E., Souza, D.O., Wajner, M. Glutaric acid stimulates glutamate binding and astrocytic uptake and inhibits vesicular glutamate uptake in forebrain from young rats. Neurochem Int 45: 1075-1086, 2004.

Quincozes-Santos, A., Rosa, R.B., Leipnitz, G., de Souza, D.F., Seminotti, B., Wajner, M., Gonçalves, C.A. Induction of S100B secretion in C6 astroglial cells by the major metabolites accumulating in glutaric acidemia type I. Metab Brain Dis 25: 191-8, 2010.

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

Rodrigues, F.S., Souza, M.A., Magni, D.V., Ferreira, A.P., Mota, B.C., Cardoso, A.M., Paim, M., Xavier, L.L., Ferreira, J., Schetinger, M.R., Da Costa, J.C., Royes, L.F., Fighera, M.R. N-acetylcysteine prevents spatial memory impairment induced by chronic early postnatal glutaric Acid and lipopolysaccharide in rat pups. PLoS One 8: e78332, 2013.

Roede, J.R., Jones, D.P. Reactive species and mitochondrial dysfunction: mechanistic significance of 4-hydroxynonenal. Environ Mol Mutagen 51: 380-390, 2010.

Rosa, R.B., Schwarzbold, C., Dalcin, K.B., Ghisleni, G.C., Ribeiro, C.A., Moretto, M.B., Frizzo, M.E., Hoffmann, G.F., Souza, D.O., Wajner, M. Evidence that 3-hydroxyglutaric acid interacts with NMDA receptors in synaptic plasma membranes from cerebral cortex of young rats. Neurochem Int 45: 1087-1094, 2004.

Rushmore, T.H., Morton, M.R., Pickett C.B. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem 266: 11632–11639, 1991.

Sauer, S.W., Okun, J.G., Fricker, G., Mahringer, A., Müller, I., Crnic, L.R., 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.

Sauer, S.W., Opp, S., Hoffmann, G.F., Koeller, D.M., Okun, J.G., Kolker, S. Therapeutic modulation of cerebral L-lysine metabolism in a mouse model for glutaric aciduria type I. Brain 134: 157-170, 2011.

Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. The metabolic and molecular bases of inherited disease, 8 ed. McGraw-Hill Inc, New York, 2001.

Seminotti, B., Leipnitz, G., Amaral, A.U., Fernandes, C.G., da Silva, L. de B., Tonin, A.M., Vargas, C.R., Wajner, M. Lysine induces lipid and protein damage and decreases reduced glutathione concentrations in brain of young rats. Int J Dev Neurosci 26: 693-698, 2008.

Seminotti, B., Fernandes, C.G., Leipnitz, G., Amaral, A.U., Zanatta, A., Wajner, M. Neurochemical evidence that lysine inhibits synaptic Na^+,K^+-ATPase activity and provokes oxidative damage in striatum of young rats in vivo. Neurochem Res 2011 36: 205-14, 2011.

Sesti, F., Tsitsilonis, O.E., Kotsinas, A., Trougakos, I.P. Oxidative stress-mediated biomolecular damage and inflammation in tumorigenesis. In Vivo 26: 395-402, 2012.

Sies, H. Oxidative stress: introductory remarks. In Sies H, editor. Oxidative Stress. Academic Press: London, 1–8, 1985.

Silva, C.G., Silva, A.R., Ruschel, C., Helegda, C., Wyse, A.T., Wannmacher, C.M., Dutra-Filho, C.S., Wajner, M. Inhibition of energy production in vitro by glutaric acid in cerebral cortex of young rats. Metab Brain Dis 15: 123-31, 2000.

Son, Y., Kim, S., Chung, H.T., Pae, H.O. Reactive oxygen species in the activation of MAP kinases. Methods Enzymol 528: 27-48, 2013.

Southorn, P.A., Powis, G. Free radicals in medicine. II. Involvement in human disease. Mayo Clin Proc 63: 390-408, 1988.

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., Mühlhausen, C. 3-Hydroxyglutaric acid is transported via the sodium-dependent dicarboxylate transporter NaDC3. J Mol Med (Berl) 85: 763-70, 2007.

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. Journal of Neurochemistry 93: 611-623, 2005.

Strauss, K.A., Morton, D.H. Type I glutaric aciduria, part 2: a model of acute striatal necrosis. Am J Med Genet C Semin Med Genet 121C: 53-70, 2003.

Strauss, K.A., Puffenberger, E.G., Robinson, D.L., Morton, D.H. Type I glutaric aciduria, part 1: natural history of 77 patients. Am J Med Genet C Semin Med Genet 121C: 38-52, 2003.

Strauss, K.A., Brumbaugh, J., Duffy, A., Wardley, B., Robinson, D., Hendrickson, C., Tortorelli, S., Moser, A.B., Puffenberger, E.G., Rider, N.L., Morton, D.H. Safety, efficacy and physiological actions of a lysine-free, arginine-rich formula to treat glutaryl-CoA dehydrogenase deficiency: focus on cerebral amino acid influx. Mol Genet Metab 104: 93-106, 2011.

Stutchfield, P., Edwards, M.A., Gray, R.G., Crawley, P., Green, A. Glutaric aciduria type I misdiagnosed as Leigh's encephalopathy and cerebral palsy. Dev Med Child Neurol 27: 514-518, 1985.

Ullrich, K., Flott-Rahmel, B., Schluff P., Musshoff, U., Das, A., Lücke, T., Steinfeld, R., Christensen, E., Jakobs, C., Ludolph, A., Neu, A., Röper, R. Glutaric aciduria type I: pathomechanisms of neurodegeneration. J Inherit Metab Dis 22: 392-403, 1999.

Varadkar, S., Surtees, R. Glutaric aciduria type I and kynurenine pathway metabolites: a modified hypothesis. J Inherit Metab Dis 27: 835-42, 2004.

Viau, K., Ernst, S.L., Vanzo, R.J., Botto, L.D., Pasquali, M., Longo, N. Glutaric acidemia type 1: outcomes before and after expanded newborn screening. Mol Genet Metab 106: 430-438, 2012.

Wajner, M., Kolker, S., Souza, D.O., Hoffmann, G.F., de Mello, C.F. Modulation of glutamatergic and GABAergic neurotransmission in glutaryl-CoA dehydrogenase deficiency. J Inherit Metab Dis 27: 825-828, 2004.

Wendel, A. Glutathione peroxidase. Meth Enzymol 77: 325–332, 1981.

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

Ziadeh, R., Hoffman, E.P., Finegold, D.N., Hoop, R.C., Brackett, J.C., Strauss, A.W., Naylor, E.W. Medium chain acyl-CoA dehydrogenase deficiency in Pennsylvania: neonatal screening shows high incidence and unexpected mutation frequencies. Pediatr Res 37: 675-8, 1995.

Zinnanti, W.J., Lazovic, J., Wolpert, E.B., Antonetti, D.A., Smith, M.B., Connor, J.R., Woontner, M., Goodman, S.I., Cheng, K.C. A diet-induced mouse model for glutaric aciduria type I. Brain 129: 899-910, 2006.

Zinnanti, W.J., Lazovic, J., Housman, C., LaNoue, K., O'Callaghan, J.P., Simpson, I., Woontner, M., Goodman, S.I., Connor, J.R., Jacobs, R.E., Cheng, K.C. Mechanism of age-dependent susceptibility and novel treatment strategy in glutaric acidemia type I. J Clin Invest 117: 3258-3270, 2007.

Zinnanti, W.J., Lazovic, J., Housman, C., Antonetti, D.A., Koeller, D.M., Connor, J.R., Steinman, L. Mechanism of metabolic stroke and spontaneous cerebral hemorrhage in glutaric aciduria type I. Acta Neuropathol Commun 2: 13, 2014.

Zschocke, J., Quak, E., Guldberg, P., Hoffmann, G.F. Mutation analysis in glutaric aciduria type I. J Med Genet 37: 177-81, 2000.

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

Yamaguchi, S., Orii, T., Yasuda, K., Kohno, Y. A case of glutaric aciduria type I with unique abnormalities in the cerebral CT findings. Tohoku J Exp Med 151:293-199, 1987.