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Efeito dos ácidos D-2-hidroxiglutárico e L-2-hidroxiglutárico sobre vários parâmetros do metabolismo energético em cérebro, músculo esquelético e músculo cardíaco de ratos

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RESUMO

As acidúrias L-2-hidroxiglutárica (LHGA) e D-2-hidroxiglutárica (DHGA) são distúrbios neurometabólicos hereditários caracterizados por extenso e severo dano cerebral, ocasionando predominantemente convulsões, coma e atrofia cerebral. Na LHGA, as lesões cerebrais ocorrem principalmente no cerebelo enquanto a maior parte do cérebro é afetada na DHGA. Além disso, hipotonia, fraqueza e hipotrofia muscular, bem como cardiomiopatia têm sido observadas nos pacientes afetados por essas acidemias orgânicas, com maior freqüência na DHGA. Bioquimicamente, ocorre acúmulo tecidual dos ácidos L-2-hidroxiglutárico (LGA) e D-2-hidróxiglutárico (DGA), respectivamente, na LHGA e na DHGA. Além disso, elevadas concentrações urinárias de lactato, 2-cetoglutarato e outros metabólitos do ciclo de Krebs têm sido descritas em pacientes acometidos por essas patologias, sugerindo uma disfunção mitocondrial. Tendo em vista que a etiopatogenia da disfunção tecidual nesses pacientes é desconhecida, o presente trabalho investigou o efeito *in vitro* dos ácidos LGA e DGA sobre diversos parâmetros do metabolismo energético celular.

Inicialmente, avaliamos o efeito dos ácidos DGA e LGA sobre a utilização de glicose e produção de CO_2 em homogeneizados e fatias de córtex cerebral. Verificamos que o DGA reduziu significativamente tanto o consumo de glicose quanto a produção de CO_2 pelo córtex cerebral, enquanto o LGA não demonstrou efeito sobre esses parâmetros. Além disso, o DGA inibiu significativamente a atividade da citocromo *c* oxidase em homogeneizado de córtex cerebral de ratos (35-95%), de forma dose-dependente, sem alterar a atividade dos demais complexos da cadeia respiratória. A inibição verificada foi do tipo acompetitiva. Por outro lado, o LGA não alterou a atividade de nenhum dos complexos enzimático estudados.

Posteriormente, avaliamos o efeito *in vitro* dos ácidos DGA e LGA sobre a atividade da creatina quinase (CK) em homogeneizado total e nas frações citosólica e mitocondrial de tecido cerebral, muscular esquelético e cardíaco de ratos. Os resultados mostraram que o DGA inibiu significativamente a atividade das isoformas mitocondrial e citosólica da CK em preparações de córtex cerebral, músculo esquelético de cardíaco. Por outro lado, tanto DGA quanto LGA inibiram seletivamente a isoforma mitocondrial em preparações de cerebelo. Estudos cinéticos mostraram um perfil não competitivo de inibição com relação à fosfocreatina para ambos os ácidos nos tecidos estudados. Além

disso, observamos também que o efeito inibitório de ambos os ácidos foi totalmente revertido por glutationa reduzida, sugerindo uma modificação causada pelos metabólitos sobre os grupos sulfidrila, essenciais para a atividade da enzima.

Nossos resultados sugerem que a inibição significativa causada pelo DGA sobre as atividades da citocromo *c* oxidase e da creatina quinase no córtex cerebral, assim como nos músculos cardíaco e esquelético poderiam explicar, ao menos em parte, a fisiopatogenia da disfunção neurológica e anormalidades estruturais no sistema nervoso central, bem como a mitocondriopatia esquelética e a cardiomiopatia presente nos pacientes afetados por DHGA. Por outro lado, é possível que a inibição seletiva da creatina quinase mitocondrial provocada pelo LGA em cerebelo possa estar associada à degeneração cerebelar característica dos pacientes com LHGA.

SUMMARY

L-2-Hydroxyglutaric aciduria (LHGA) and D-2-hydroxyglutaric aciduria (DHGA) are inherited metabolic disorders characterized by severe neurological damage, which leads to seizures, comma and cerebral atrophy. Brain lesions occur in the cerebellum (LHGA) and in the whole brain (DHGA). Hypotonia and cardiomyopathy are also observed in patients affected by these organic acidemias, particularly with DHGA. Biochemically, LHGA and DHGA are characterized by tissue accumulation and high urinary excretion of L-2-hydroxyglutaric acid (LGA) and D-2-hydroxyglutaric acid (DGA), respectively. In addition, increased urinary excretion of lactate, alpha-ketoglutarate and other intermediates of the Krebs cycle has been also described in patients with these disorders. Since the pathophysiology of the tissue damage in these diseases is unknown, the present study investigated the in vitro effects of LGA and DGA on various parameters of cellular energy metabolism.

We initially evaluated the effects of LGA and DGA on glucose utilization and CO_2 production in homogenates and slices of cerebral cortex. We observed that DGA significantly reduced both glucose uptake and CO_2 formation by the cerebral cortex, whereas LGA did not affect these parameters. Furthermore, DGA significantly inhibited the activity of cytochrome c oxidase in cortical homogenates (35-95 %) in a dose-dependent fashion and in an acompetitive way, without altering the other activities of the mitochondrial respiratory chain complexes. In contrast, LGA did not alter any of the activities studied.

We further studied the in vitro effect of DGA and LGA on creatine kinase (CK) activity in total homogenates (tCK), as well as on cytosolic and mitochondrial preparations in brain, skeletal muscle and cardiac muscle of rats. The results demonstrated that DGA significantly inhibited the activities of the mitochondrial and cytosolic isoforms from cerebral cortex, skeletal muscle and cardiac muscle. Furthermore, LGA and DGA selectively inhibited the mitochondrial isoform in cerebellum in a noncompetitive manner relative to phosphocreatine. Finally, we observed that this inhibition was prevented by reduced glutathione, indicating the involvement of thiol groups of the enzyme.

Our results suggest that the strong inhibition of COX activity associated to an inhibition of creatine kinase by DGA, as well as an inhibition of the mitochondrial CK activity by LGA in cerebellum may impair brain celullar energy metabolism which could be at least partially responsible for the neurological dysfunction present in patients affected by these pathologies.

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LISTA DE ABREVIATURAS

ADP: adenosina 5'-difosfato

AMP: adenosina 5'-monofosfato

ATN: translocador de nucleosídeos de adenina

ATP: adenosina 5'-trifosfato

CK: creatina quinase

CoQ: coenzima Q

COX: citocromo *c* oxidase

CR: cadeia respiratória

Cr: creatina

CS: sítios de contato

Cy-CK: creatina quinase citosólica

DGA: Ácido D-2-hidroxiglutárico

DHGA: Acidúria D-2-hidroxiglutárica

EDTA: ácido etilenodiamino-tetracético

EIM: erros inatos do metabolismo

FAD: flavina adenina dinucleotídeo

$FADH_2$: flavina adenina dinucleotídeo (forma reduzida)

FMN: flavina mononucleotídeo

GABA: ácido gama-aminobutírico

GTP: trifosfato de guanosina

HEPES: ácido N-2-hidroxietilpiperazina

LCR: líquor

LHGA: Acidúria L-2-hidroxiglutárica

LGA: Ácido L-2-hidroxiglutárico

L-NAME: Nω-nitro-L-arginine methyl ester

Mi-CK: creatina quinase mitocondrial

IRM: imagem de ressonância magnética

NAD^+: nicotinamida adenina dinucleotídeo (forma oxidada)

NADH: nicotinamida adenina dinucleotídeo (forma reduzida)

NO: óxido nítrico

OXPHOS: fosforilação oxidativa

PCr: fosfocreatina

PN: peroxinitrito

PTP: poros de transição de permeabilidade

ROO⁻: radical peroxila

ROS: espécies reativas de oxigênio

SNC: sistema nervoso central

TCA: ácido tricloroacético

TRIS: tris(hidroximetil)-aminoetano

I. INTRODUÇÃO

I. 1. Erros inatos do metabolismo

I. 1. 1. Histórico

O termo erros inatos do metabolismo (EIM) foi proposto em 1908 pelo inglês Archibal Garrod, referindo-se à alcaptonúria, cistinúria, pentosúria e albinismo. Fundamentado nas recém descobertas leis de Mendel e baseado em suas observações de que essas doenças eram mais freqüentes entre indivíduos da mesma família, embora seus pais e outros parentes fossem saudáveis, Garrod propôs um modelo de herança genética autossômica recessiva para essas patologias. Também observou que alguns dos pacientes acometidos por essas enfermidades excretavam na urina quantidades aumentadas de determinadas substâncias intermediárias do metabolismo. A partir disso, Garrod propôs que o acúmulo dessas substâncias era decorrente de um bloqueio parcial ou total de determinadas rotas metabólicas, devido à deficiência da atividade de uma enzima responsável por uma reação específica para cada caso. Posteriormente, foi demonstrado que cada gene exerce influência sobre a síntese de uma determinada enzima. Baseado nessa demonstração, o conceito inicial de EIM foi reformulado por Beadle & Tatum em 1941, sob a hipótese "um gene - uma enzima". Esse conceito expressava que toda reação bioquímica estava sob controle de um único gene e que a mutação desse, resultando na modificação estrutural e funcional da enzima, ocasionava uma alteração da capacidade celular de realizar uma determinada reação química. O primeiro defeito enzimático causador de uma doença genética foi demonstrado por Gibson em 1948. Entretanto, a hipótese de Garrod só foi confirmada em 1958, quando La Du e colaboradores comprovaram a deficiência da oxidase do ácido homogentísico no fígado de um paciente com alcaptonúria.

I. 1. 2. Conceito e classificação dos erros inatos

Os EIM são alterações genéticas que se manifestam pela síntese de uma proteína anômala, geralmente uma enzima, podendo expressar-se na troca da seqüência primária, levando a alterações estruturais, com modificações nas suas propriedades cinéticas, diminuindo sua estabilidade biológica e/ou facilitando sua degradação *in vivo*. Pode também ocorrer uma diminuição ou mesmo uma ausência na síntese protéica. A resultante de qualquer dessas alterações é uma diminuição na atividade da enzima envolvida ou a inativação da mesma. Como conseqüência da ausência ou deficiência severa da atividade

enzimática podem ocorrer o acúmulo de substâncias normalmente presentes em pequenas quantidades (A), a deficiência de produtos intermediários críticos (B), a deficiência de produtos finais específicos (C) ou ainda o excesso de produtos nocivos de vias metabólicas acessórias (D), como mostra a figura I.1.
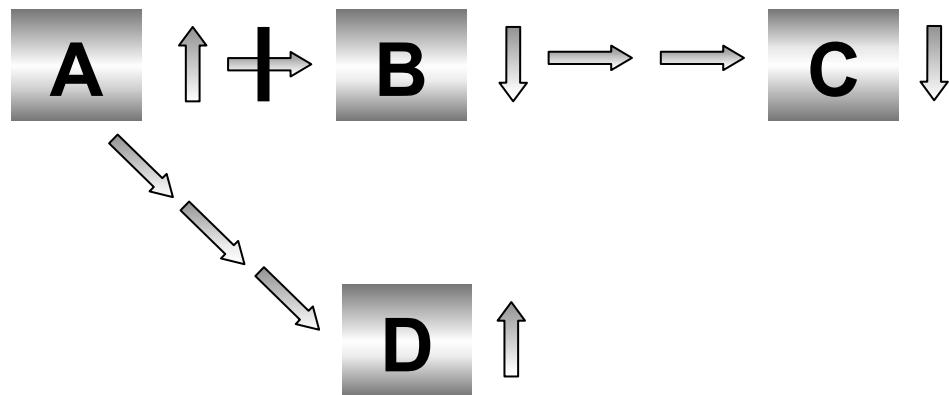
Figura I.1. Via metabólica com deficiência na atividade de uma enzima.

Além das enzimas, outras proteínas também podem estar envolvidas no defeito básico dos EIM, como proteínas de transporte, receptores, hormônios peptídicos, imunoglobulinas, colágeno, fatores de coagulação, fatores de transcrição, dentre outras.

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

a) Desordens de transporte: Envolvem proteínas transportadoras de moléculas orgânicas ou inorgânicas no rim ou no intestino. Exemplos: deficiência de dissacaridases e defeito no transporte de magnésio;

b) Desordens de armazenamento, degradação e secreção: Envolvem proteínas relacionadas com o aparelho de Golgi ou lisossomas na sua grande maioria, provocando acúmulo de macromoléculas em tecidos específicos. Exemplos: doenças lisossômicas de depósito, glicogenoses e cistinose;

c) Desordens de síntese: Envolvem a síntese de proteínas com funções hormonais, de defesa imunológica, estruturais, etc. Exemplos: hiperplasia adrenal congênita, hipogamaglobulinemia;

d) Desordens do metabolismo intermediário: Envolvem deficiências enzimáticas das rotas de metabolização de moléculas pequenas (aminoácidos, glicídios, neurotransmissores, etc.), podendo comprometer rotas importantes como os ciclos dos ácidos tricarboxílicos e da uréia, ou rotas relacionadas a essas. O bloqueio produz o acúmulo de substrato da enzima deficiente, bem como dos metabólitos produzidos a partir desse, além da deficiência do produto final da rota, caso não possa ser suprido por outra via metabólica. O acúmulo de metabólitos tóxicos pode causar outras alterações bioquímicas secundárias e danos nos tecidos. Como esses produtos são liberados na circulação, podem prejudicar outros tecidos, sendo finalmente excretados na urina. Essas desordens constituem o maior grupo dos EIM e têm como exemplos as acidúrias orgânicas, as aminoacidopatias, as desordens do metabolismo das purinas, da frutose, galactose e pentose, e outros.

Outra classificação de EIM leva em conta a área do metabolismo afetada, com especial ênfase para os metabólitos acumulados (tabela I.1.).

Tabela I.1. Classificação dos erros inatos do metabolismo (EIM) (baseada em Scriver *et al*, 2001).

EIM dos carboidratos
EIM dos aminoácidos
EIM dos ácidos orgânicos
EIM das purinas e pirimidinas
EIM das lipoproteínas
EIM das porfirinas e do heme
EIM das bilirrubinas
EIM dos metais
Desordens dos peroxissomas
Doenças lisossomais
EIM dos hormônios
EIM das vitaminas
EIM do sangue e dos tecidos hematopoiéticos
EIM dos sistemas de transporte de membrana
EIM dos mecanismos de imunidade de defesa
EIM do tecido conjuntivo e da pele
EIM do intestino

I. 1. 3. Incidência

Os EIM individualmente são considerados raros, porém, quando consideramos em conjunto, sua incidência é de 1: 500 a 1: 1.000 recém-nascidos vivos. Em triagem neonatal em massa para alguns EIM observa-se positividade de 1: 3.803 (Pollitt *et al*, 1997). A prevalência de alguns EIM em populações brasileiras é como segue: fenilcetonúria de 1: 12.000 a 1: 15.000, doença da urina do xarope do bordo de 1: 43.000 (Schmidt *et al*, 1987; Camargo Neto *et al*, 1993) e deficiência de biotinidase de 1: 125.000 (Pinto *et al*, 1998).

I. 1. 4. Aspectos clínicos

Embora a sintomatologia dos EIM seja bastante variável, existem alguns sintomas e sinais clínicos que aparecem com maior freqüência nesses distúrbios. As tabelas I.2. e I.3. mostram os achados clínicos mais freqüentes nos EIM.

Tabela I.2. Principais manifestações clínicas dos erros inatos do metabolismo no período neonatal (Burton, 1987).

Retardo no crescimento
Dificuldade alimentar
Vômitos
Diarréia
Letargia ou coma
Hipotonicidade
Convulsões
Dificuldade respiratória e apnéia
Icterícia
Hepatomegalia
Odor anormal da pele
Dismorfias
Anormalidades oculares
Cabelos anormais
Macroglossia

Tabela I.3. Manifestações clínicas dos EIM (adaptado de Wappner, 1993).

Clínica e Laboratório	Doenças Lisossomais	Doenças Peroxissomais	Aminoacidopatias	Acidúrias Orgânicas	Defeitos do Ciclo da uréia	Intolerância aos açúcares	Doenças de depósito do glicogênio	Doenças Mitocondriais	Defeitos de oxidação de Ácidos Orgânicos	Acidemias Láticas
Episódico	-	-	++	++	++	++	++	+	++	-
Dificuldade Alimentar	+	+	++	+	++	+	+	+	+	+
Odor Anormal	-	+	+	-	-	-	-	-	-	-
Letargia, Coma	-	-	+	+	++	+	+	+	+	-
Convulsões	++	+	+	+	+	+	+	+	+	+
Regressão do desenvolvimento	++	++	-	+	+	-	-	+	-	-
Hepatomegalia	++	+	+	+	+	+	++	+	+	-
Hepatoesplenomegalia	++	+	-	-	-	-	+	-	-	-
Esplenomegalia	+	-	-	-	-	-	-	-	-	-
Hipotonia	+	+	+	+	+	+	+	+	+	++
Cardiomiopatia	+	-	-	+	-	-	+	++	+	-
Faces Grotescas	+	-	-	-	-	-	-	-	-	-
Hipoglicemia	-	-	+	+	-	++	++	+	++	-
Hiperglicemia	-	-	-	++	-	-	-	-	-	-
Acidose metabólica	-	-	+	++	+	++	++	+	+	+
Alcalose metabólica	-	-	-	-	++	-	-	-	-	-
Hiperamonemia	-	-	+	+	++	-	-	-	+	-
Hiperlacticemia	-	-	-	++	-	+	++	++	+	++
Cetose	-	-	+	+	+	+	+	-	-	-

++ geralmente presente
+ pode estar presente
- normalmente não está presente

I. 1. 5. *Diagnóstico laboratorial*

Inicia-se com a pesquisa de metabólitos urinários e plasmáticos através de testes de triagem urinária para EIM (tabela I.4.) e da cromatografia qualitativa e semiquantitativa para aminoácidos no sangue e urina. A cromatografia plasmática quantitativa de aminoácidos e a cromatografia qualitativa de ácidos orgânicos na urina são solicitadas de acordo com os resultados da triagem e indicação clínica. Concomitantemente, é realizada a avaliação sangüínea de rotina na qual incluem-se hemograma, gasometria venosa, determinação de sódio (Na^+), potássio (K^+), cloreto (Cl^-), glicemia de jejum, transaminases hepáticas, colesterol total e frações, triglicérides, ácido úrico, lactato, piruvato e amônia.

Na presença de acidose metabólica, deve ser sempre realizado o cálculo do hiato aniônico através da fórmula: $(Na^+ + K^+) - (HCO_3^- + Cl^-)$, quando o valor for acima de 16 existe a suspeita de acidúria orgânica. Nos EIM que cursam com crises de descompensação metabólica, é crucial para o diagnóstico que os exames (gasometria venosa, Na^+, K^+, Cl^-, glicemia, lactato, piruvato e amônia) sejam colhidos durante a descompensação, antes da introdução das medidas terapêuticas de urgência. A tabela I.5. traz os achados laboratoriais mais freqüentes nos pacientes com EIM.

Tabela I.4.: Testes de triagem urinária para EIM e as doenças que podem ser detectadas.

Teste	Doenças detectáveis
Reação de Benedict	Galactosemia. Intolerância à frutose. Alcaptonúria e Síndrome de Lowe. Positivo também para: diabete mellitus, glicosúria renal, doença de Fanconi, deficiência de lactase, pentosúria, ingestão excessiva de vitamina C, uso de sulfonamidas, tetraciclinas, cloranfenicol e ácido ρ-aminosalicílico.
Reação do cloreto férrico	Fenilcetonúria. Tirosinemia. Tirosinose. Histidinemia. Alcaptonuria. Doença do xarope do bordo. Hiperglicinemia. Positivo também para: feocromocitoma, síndrome carcinóide, cirrose hepática, tirosinemia transitória, icterus, excreção de iodoclorohidroxiquinina e de metabólitos da L-dopa, acidose pirúvica, excreção do ácido acetoacético, de salicilatos, de antipirina, de derivados de fenotiazina, de ácido vanílico e má-absorção de metionina, melanina, acidose lática e excreção de isoniaxida.
Reação da dinitrofenilhidrazina	Fenilcatonúria. Doença do xarope do bordo. Tirosinose. Histidinemia. Má-absorção de metionina. Hiperglicinemia. Glicogenoses I, III, V e VI. Acidose láctica e Acidose pirúvica.
Reação do nitrosonaftol	Tirosinose. Tirosinemias hereditárias. Tirosinemia transitória. Disfunção hepática severa. Frutosemia e Galactosemia.
Reação da ρ-nitroanilina	Acidúria metilmalônica
Reação do brometo de cetil-trimetil-amônio (CTMA)	Mucopolissacaridoses. Positivo também para: síndrome de Marfan, mastocitose, artrite reumatóide, creatinismo e carcinomatose.
Reação do cianeto-nitroprussiato	Homocistinúria. Cistinúria
Reação do axul de toluidina	Mucopolissacaridoses. Pode ser positiva para: síndrome de Marfan, mastocitose, artirte reumatóide, creatinismo e carcinomatose.
Reação de Erlich	Casos de Porfiria.

Tabela I.5. Achados laboratoriais comuns associados a EIM no período neonatal (Burton, 1987).

Acidose metabólica
Hipoglicemia
Hiperamonemia
Transaminases elevadas
Substâncias redutoras na urina
Cetonúria
Neutropenia
Trombocitopenia
Anemia
Linfócitos vacuolados no esfregaço periférico

I. 2. Acidúrias Orgânicas

As acidúrias ou acidemias orgânicas são distúrbios do metabolismo de aminoácidos, glicídios ou lipídios, causados por um bloqueio metabólico devido à deficiência severa da atividade de uma enzima. Esse grupo de doenças é caracterizado por acúmulo de ácidos orgânicos, seus ésteres e conjugados, em tecidos e fluidos corpóreos e principalmente na urina.

A prevalência das acidúrias orgânicas na Holanda, um país modelo para o estudo dos EIM, é tida como a mais aproximada da realidade, estimando-se para 1 para cada 2200 habitantes, enquanto na Arábia Saudita, onde a taxa de consangüinidade é elevada, é de pelo menos 1:740 nascimentos (Rashed *et al*, 1994; Hoffmann, 1994). Relativamente a outros grupos de EIM, as acidemias orgânicas são consideradas as mais freqüentes doenças metabólicas em crianças severamente enfermas (Chalmers *et* al, 1980; Wajner *et* al, 1986) e dos mais freqüentes grupos de enfermidades hereditárias do metabolismo (Hoffmann, 1994). As acidúrias orgânicas mais comumente encontradas são as acidúrias metilmalônica, propiônica, deficiência de aconitase, 3-hidroxi-3-metil glutárica e acidúria glutárica Tipo I (deficiência da glutaril CoA desidrogenase).

O quadro clínico neonatal caracteriza-se por hipotonia, acidose metabólica grave e de difícil controle, vômitos, hipoglicemia ou hiperglicemia, letargia e coma. As manifestações clínicas na infância incluem dificuldade de crescimento, macrocrania, hipotonia, atraso no desenvolvimento neuropsicomotor, crises de descompensação associadas à infecção ou estresse, síndrome similar à "Reye", convulsões associadas com hipoglicemia, hiperglicemia e acidose tubular renal.

O diagnóstico é realizado através da detecção de ácidos orgânicos na urina por cromatografia gasosa associada à espectrometria de massa.

O tratamento no período neonatal consiste em suspender a ingestão de proteína, fazer a correção da acidose e tratar as causas subjacentes (infecção) até a confirmação do diagnóstico. Em seguida deve ser restabelecida gradualmente a ingestão protéica, promovendo assim o anabolismo. A longo prazo devem ser utilizadas fórmulas metabólicas (leite) isentas do(s) aminoácido(s) precursor(es) dos ácidos orgânicos. Existe grande variabilidade com relação às manifestações clínicas e resposta ao tratamento.

I. 2. 1. Acidúrias 2-hidroxiglutáricas

O ácido 2-hidroxiglutárico é um metabólito normalmente encontrado, em pequenas concentrações, na urina humana podendo ocorrer nas configurações D e L. Dois tipos de acidemias hidroxiglutáricas têm sido descritos: as acidúrias L-2-hidroxiglutárica (LHGA) e D-2-hidroxiglutárica (DHGA), caracterizadas pelo acúmulo dos ácidos L-2-hidroxiglutárico (LGA) ou do isômero D-2-hidroxiglutárico (DGA), respectivamente. São desordens metabólicas distintas, transmitidas de forma autossômica recessiva (van der Knaap *et al*, 1999a,b).

I. 2. 1. 1. Acidúria D-2-hidroxiglutárica

I. 2. 1. 1. 1. Conceito e freqüência

A DHGA foi primeiramente descrita em 1980 por Chalmers e colaboradores. A DHGA é um erro inato do metabolismo pertencente ao subgrupo das desordens de ácidos orgânicos denominadas acidemias orgânicas cerebrais devido à apresentação clínica ser predominantemente cerebral (Hoffman *et al*, 1994). A DHGA parece ser mais rara que a LHGA e atualmente pelo menos 26 pacientes foram descritos (van der Knaap *et al*, 1999a,b; Wajner *et al*, 2002).

I. 2. 1. 1. 2. Aspectos clínicos e neuropatológicos

Existe uma grande variabilidade quanto à severidade com que os sinais clínicos da DHGA podem se manifestar, apresentando-se desde uma disfunção cerebral severa, com sério retardo no desenvolvimento e morte precoce, até casos nos quais ocorrem apenas alterações gastrintestinais ou mesmo onde nenhuma manifestação clínica importante é observada. Entretanto, uma análise aprofundada dos dados clínicos, bioquímicos e neuropatológicos coletados até o momento revelou que o quadro clínico apresentado pelos

pacientes com DHGA permite a classificação da doença em dois fenótipos distintos nos quais as manifestações clínicas são relativamente homogêneas.

O fenótipo severo caracteriza-se por encefalopatia epilética de início neonatal ou na primeira infância, hipotonia, movimentos distônicos ou coreoatetóticos, deficiência visual, e severo retardo no desenvolvimento psicomotor. Além disso, cardiomiopatia tem sido freqüentemente observada. Exames de neuroimagem revelaram atraso na maturação cerebral, moderado aumento dos ventrículos laterais freqüentemente combinada de leve aumento do espaço subaracnóideo e presença de cistos subependimais. Um terço dos pacientes morre durante a infância (van der Knaap *et al*, 1999a).

A variante suave da DHGA é muito mais variável quanto à sintomatologia clínica, sendo hipotonia, retardo no desenvolvimento e macrocefalia achados bastante freqüentes. Ventriculomegalia e sinais de atraso na maturação cerebral foram também observados, embora alguns pacientes não apresentem alterações neurológicas graves (van der Knaap *et al*, 1999a).

I. 2. 1. 1. 3.   *Aspectos bioquímicos*

Bioquimicamente, DHGA é caracterizada pelo acúmulo tecidual de DGA, com elevada excreção urinária do ácido em todos os pacientes (Gibson *et al*, 1993a). As concentrações de DGA no plasma e líquor (LCR) apresentam-se na faixa de micromolar, com grande variação entre os indivíduos afetados. Não tem sido encontrada correlação entre os níveis urinários, plasmáticos ou liquóricos de DGA e a severidade da doença, embora os maiores níveis (plasma, acima de 750 μM, LCR acima de 300μM) tenham sido observados nos pacientes com fenótipo severo (van der Knaap *et al*, 1999a). As concentrações intracelulares de DGA ainda são desconhecidas, embora se acredite que possam ser maiores do que os níveis plasmáticos desse metabólito (Hoffmann *et al*, 1994). Além disso, elevadas concentrações de 2-cetoglutarato na urina e GABA no LCR têm sido descritas (van der Knaap 1999a,b; Gibson *et al*, 1993a).

O defeito bioquímico da DHGA é ainda desconhecido e pouco se sabe sobre o papel do DGA no metabolismo. O DGA é um metabólito intermediário em diversas rotas bioquímicas (Gibson *et al*, 1993b, Nyhan *et al*, 1995). Duas rotas bioquímicas têm sido descritas em mamíferos para o metabolismo do DGA: (a) a conversão do 2-cetoglutarato a DGA pela enzima mitocondrial D-2-hidroxiglutarato desidrogenase (Tubbs & Greville, 1961) e (b) a conversão do 2-cetoglutarato em DGA pela enzima D-2-hidroxiglutarato transidrolase usando o semialdeído succínico, um catabólito imediato do GABA, como

aceptor de hidrogênio (Kaufman *et al*, 1988) (figura I.2.). Portanto, uma deficiência na conversão do 2-cetoglutarato a DGA elevaria o GABA e intermediários do ciclo do ácido cítrico. Entretanto, não foi comprovada, até o momento, nenhuma ligação entre as enzimas mencionadas e a DHGA humana.
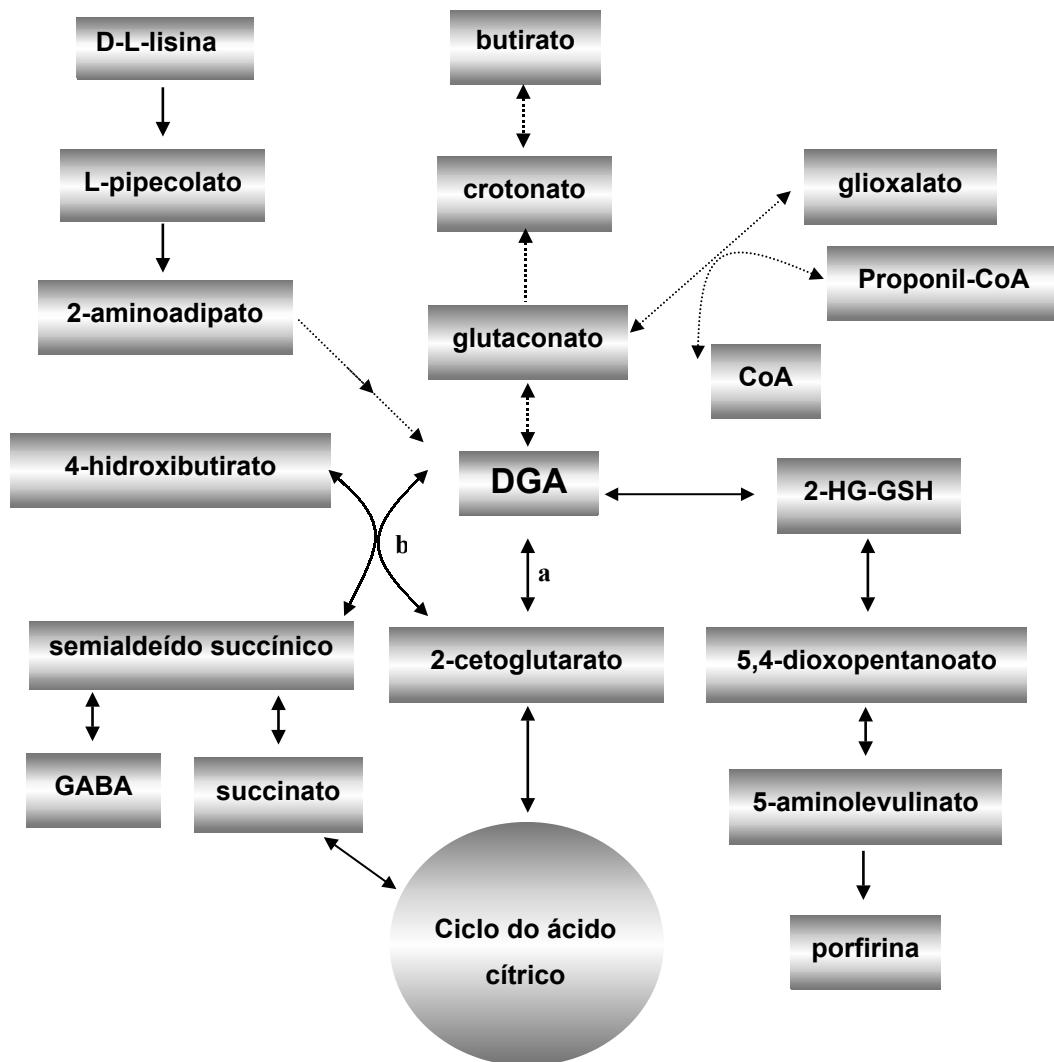
*Figura I.2.*Rotas metabólicas envolvendo o DGA.
Linhas sólidas: reações em mamíferos; linhas pontilhadas: reações documentadas em bactérias (a): D-2-hidroxiglutarato desidrogenase; (b) D-2-hidroxiglutárico transidrolase; 2-HG-GSH: 2-hidroxiglutaril glutationa; CoA: coenzima A, GABA: ácido gama-aminobutírico.

O perfil urinário de ácidos orgânicos dos pacientes afetados por DHGA mostra freqüente elevação na excreção de 2-cetoglutarato, algumas vezes acompanhada por elevada excreção de outros metabólitos intermediários do ciclo o ácido cítrico. Contudo ainda se desconhece se essas anormalidades refletem um distúrbio primário ou secundário do ciclo de Krebs ou uma disfunção secundária da cadeia respiratória (van der Knaap *et al*, 1999a).

I. 2. 1. 1. 4. *Aspectos fisiopatológicos*

A fisiopatologia da sintomatologia clínica na DHGA é ainda desconhecida. Os sinais de disfunção do sistema nervoso central são dominantes, mas existem evidências de que a hipotonia possa ser, pelo menos em parte, explicada por patologias musculares. Estudos histopatológicos realizados em biópsias de músculo esquelético em dois pacientes afetados mostraram marcado aumento de glicogênio nas miofibrilas no primeiro e lesões inespecíficas caracterizadas por depósitos de matriz extracelular entre as fibras musculares e muitas fibras atróficas no segundo (van der Knaap *et al*, 1999a; Eeg-Olofsson, 2000). Contudo, ainda é incerta a contribuição da patologia muscular na DHGA.

Estudos recentes tem contribuído para elucidar os mecanismos fisiopatológicos da disfunção cerebral nos pacientes com DHGA. Kölker e colaboradores demonstraram que DGA ativa *in vitro* receptores NMDA glutamatérgicos, promovendo dano neuronal por excitotoxicidade. Além disso, foi demonstrado que o DGA é capaz de inibir o complexo V da cadeia respiratória. Os autores sugerem que tal inibição poderia exacerbar o dano neuronal mediado pela excitotoxicidade produzida por esse ácido (Kölker *et al*, 2002).

I. 2. 1. 2. **Acidúria L-2-hidroxiglutárica**

I. 2. 1. 2. 1. *Conceito e freqüência*

A LHGA é um raro erro inato do metabolismo, do grupo das acidemias orgânicas cerebrais, tendo sido inicialmente descrito por Duran, em 1980. Em 1992, Barth e colaboradores definiram a partir de oito pacientes as características clínicas, bioquímicas e neurológicas dessa doença. Atualmente, há pelo menos 50 casos descritos no mundo (Barth *et al*, 1992; Barth *et al*, 1993; Divry *et al*, 1993; Wilcken *et al*, 1993; Chen *et al*, 1996; Diogo *et al*, 1996; De Klerk *et al*, 1997; Barbot *et al*, 1997).

I. 2. 1. 2. 2. *Aspectos clínicos e neuropatológicos*

A LHGA apresenta um quadro clínico bastante característico, bem como achados cerebrais típicos à imagem de ressonância magnética (IMR). A doença é crônica, lenta e progressiva, sem flutuação ou deterioração agudas (Barbot *et al*, 1997). Em geral, o início das manifestações clínicas ocorre durante o primeiro ano de vida, com moderado retardo motor, algumas vezes com a ocorrência de convulsões febris. Macrocefalia de grau moderado está presente em aproximadamente 50% dos pacientes, podendo algumas vezes ser uma das primeiras manifestações da doença (Diogo *et al*, 1996, Barbot *et al*, 1997). No

segundo ano de vida, os pacientes geralmente apresentam deficiência mental progressiva, macrocefalia não congênita e progressiva atrofia cerebelar, variando em severidade, mas presente em todos os pacientes. O quadro clínico da maioria dos pacientes afetados por LHGA caracteriza-se por retardo psicomotor, progressiva ataxia combinada com leucoencefalopatia subcortical, atrofia cerebelar e sinais de alterações nos gânglios da base à IMR. (Barth *et al*, 1992; Barth *et al*, 1993; Divry *et al*, 1993; Wilcken *et al*, 1993, De Klerk *et al*, 1997; Barbot *et al*, 1997; D'Incerti *et al*, 1998). Sintomas extrapiramidais e piramidais, e convulsões são achados clínicos freqüentes.

I. 2. 1. 2. 3.   *Aspectos bioquímicos*

Bioquimicamente, a LHGA caracteriza-se pelo acúmulo persistente de LGA no plasma e LCR, bem como por elevada excreção urinária do ácido. Outro achado bioquímico consistente é o aumento das concentrações de lisina no plasma e LCR dos pacientes afetados (Barbot *et al*, 1997). O ácido lático também tem suas concentrações séricas e urinárias aumentadas em alguns pacientes. Não tem sido observada qualquer correlação entre a severidade dos achados clínicos e as quantidades urinárias excretadas de LGA (Barbot *et al*, 1997).

O bloqueio metabólico envolvido na LHGA é ainda desconhecido. O catabolismo do L-2-hidroxiglutarato vem sendo estudado em tecido hepático e uma nova enzima, a L-2-desidrogenase, foi identificada (Jansen & Wanders, 1993). Entretanto, nenhuma deficiência enzimática tem sido comprovada nos pacientes afetados.

I. 2. 2. *Diagnóstico e tratamento das acidúrias 2-hidroxiglutáricas*

Até o momento, nenhuma deficiência enzimática específica foi relacionada às acidúrias L- e D-2-hidroxiglutáricas. Dessa forma, o diagnóstico depende da análise dos ácidos orgânicos urinários. A triagem de rotina por cromatografia gasosa acoplada a espectrometria de massa para ácidos orgânicos revela um grande pico de ácido 2-hidroxiglutárico. Uma análise especial para a determinação da configuração absoluta dos enantiômeros é necessária para distinguir os isômeros L e D, sendo essa diferenciação fundamental ao diagnóstico (Sewell *et al*, 1998; Zafeiriou *et al*, 2001).

Nenhuma terapia específica está disponível para essas doenças metabólicas (Barbot *et al*, 1997).

I. 3. Metabolismo energético

I. 3. 1. Metabolismo energético cerebral

A glicólise e a fosforilação oxidativa são particularmente importantes no cérebro para a produção de energia, porque a glicose é o principal composto energético utilizado pelo sistema nervoso central (SNC) e, no cérebro, a fosforilação oxidativa fornece mais de 95% do ATP sintetizado. Por outro lado, a oxidação de corpos cetônicos ocorre no cérebro de forma efetiva no jejum (Siesjo, 1978).

Em condições onde as concentrações plasmáticas de glicose são normais, o conteúdo de glicose cerebral é de aproximadamente 2-3 μmol/g de tecido (Cunningham *et al*, 1986, Manson *et al*, 1992), havendo pouca variação de uma região para outra (Cunningham *et al*, 1986). O transporte da glicose através da barreira hematoencefálica, bem como através das membranas neuronais e das células gliais é muito rápido. Sendo assim, o metabolismo cerebral da glicose é mais regulado por sua fosforilação do que por seu transporte (Lund-Andersen, 1979). A reserva energética cerebral (glicogênio) é extremamente pequena em relação a sua elevada atividade metabólica, de modo que a função normal do SNC requer o suprimento contínuo de glicose a partir da circulação (Erecinska & Silver, 1994). O glicogênio está localizado principalmente, mas não exclusivamente, nos astrócitos (Cataldo & Broadwell, 1986). No cérebro mais de 95% da glicose é convertida em CO_2 e água enquanto uma pequena fração é convertida em lactato ou segue outras rotas metabólicas (Hawkins *et al*, 1974; Siesjo, 1978).

Lactato e piruvato podem ser transportados através da barreira hematoencefálica por mecanismos específicos saturáveis utilizando transportadores para ácidos monocarboxílicos. Tanto lactato como piruvato podem ser prontamente oxidados nas células cerebrais. O lactato tem sido identificado como um importante substrato durante o período neonatal (Medina, 1985).

Em estados cetóticos, onde a produção e, conseqüentemente, as concentrações séricas de corpos cetônicos estão elevadas, D-β-hidroxibutirato e acetoacetato podem substituir, pelo menos em parte, a glicose, podendo ser oxidados pelo cérebro em quantidades significativas (Owen *et al*, 1967). Nos recém-nascidos, acetoacetato é metabolisado pelo cérebro com a mesma velocidade que a glicose, enquanto adultos metabolisam a glicose mais rapidamente (Spitzer, 1973). O tecido cerebral pode oxidar

ácidos graxos, mas normalmente, somente em pequena escala (Abood & Geiger, 1955), o mesmo ocorrendo para os aminoácidos (Lajtha & Toth, 1961).

Tendo em vista que a fosforilação oxidativa é responsável pela quase totalidade do ATP produzido no SNC, a regulação da respiração mitocondrial é central para o metabolismo e funções cerebrais.

Outro mecanismo cerebral importante na manutenção dos níveis constantes de ATP é o sistema catalisado pela creatina quinase. O cérebro de mamíferos contém uma reserva energética adicional na forma de sistema fosfocreatina/creatina. O conteúdo total de nucleotídeos de adenina (ATP + ADP + AMP) está em torno de 3μmol/g de tecido. A concentração de ATP excede em 10 vezes a de ADP e em quase 100 vezes a de AMP. Creatina e fosfocreatina totalizam 10-14 μmol/g de tecido e estão presentes na proporção de 1:1. (Erecinska & Silver, 1994).

I. 3. 2. Glicólise

A degradação da glicose para produção de energia está presente em todos os seres vivos, desde a mais antiga e simples bactéria até os complexos organismos multicelulares. A glicólise, também conhecida como via de Ebden-Meyerhof, é a rota metabólica pela qual a glicose é convertida, via frutose-1,6-bifosfato, a piruvato, com a geração de 2 moles de ATP/mol de glicose. Essa seqüência de 10 reações enzimáticas tem papel fundamental no metabolismo energético por fornecer parte da energia utilizada pela maioria dos organismos. Em condições aeróbicas, o piruvato formado pela glicólise é oxidado a CO_2 e água pelo ciclo do ácido cítrico seguido da fosforilação oxidativa. Entretanto, sob condições anaeróbicas, o piruvato é prontamente convertido a um produto final reduzido (Voet & Voet, 1995). As enzimas da via glicolítica estão localizadas no citosol (Voet & Voet, 1995).

I. 3. 3. Ciclo de Krebs

Nos organismos aeróbios, o piruvato resultante da glicólise entra na mitocôndria e sofre descarboxilação e desidrogenação pela ação de um complexo enzimático denominado de piruvato desidrogenase, formando uma molécula de NADH e acetil-CoA que é oxidado no Ciclo de Krebs (figura I.3.).
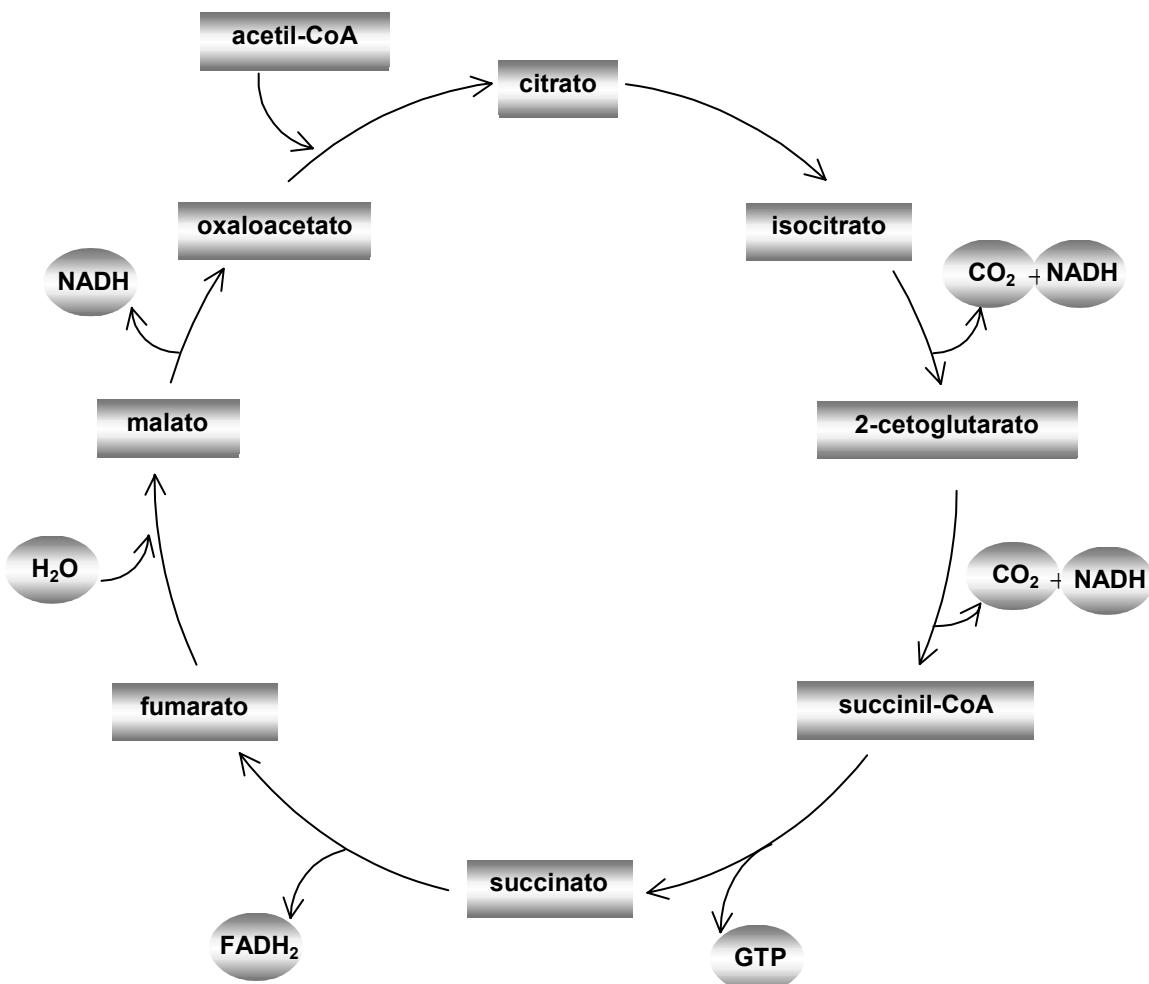
Figura I.3. Ciclo do ácido cítrico

O acetil-CoA é oxidado por uma série de reações originando GTP, CO_2 e nucleotídios reduzidos (3 NADH e 1 $FADH_2$). O controle do ciclo de Krebs ocorre principalmente nas reações catalisadas pelas enzimas isocitrato desidrogenase, α-cetogluratato desidrogenase e succinato desidrogenase que são inibidas por excesso de NADH. Assim, quando os nucleotídeos reduzidos (NADH e $FADH_2$) não são oxidados na cadeia respiratória por bloqueio dessa, o ciclo de Krebs estará inibido, bem como a transformação de piruvato em acetil-CoA. O piruvato é então convertido a lactato que se acumula nos tecidos (Voet & Voet, 1995).

I. 3. 4. *Cadeia respiratória e fosforilação oxidativa*

A energia necessária para geração de ATP é gerada através da fosforilação oxidativa (OXPHOS). A OXPHOS é um processo que requer a ação orquestrada de cinco complexos enzimáticos distribuídos de forma especial na membrana mitocondrial interna, que constituem a chamada cadeia respiratória (CR). Os elétrons oriundos do NADH e $FADH_2$, provenientes do ciclo de Krebs e de outras reações catalisadas por desidrogenases,

são transferidos para a CR, tendo o oxigênio molecular como aceptor final. O processo de transferência de elétrons é acoplado à translocação de prótons através da membrana mitocondrial interna e à síntese endoergônica de ATP, tendo como força motriz a energia armazenada primariamente como gradiente eletroquímico de prótons (Babcock & Wikstöon, 1992; Voet & Voet, 1995). Os primeiros dois eventos ligado à respiração, transferência de elétrons e bombeamento de prótons são realizados pela cadeia respiratória. Os complexos enzimáticos da CR compreendem a maior parte das proteínas embebidas na membrana mitocondrial interna. Cada complexo é constituído de vários componentes protéicos que são associados com uma variedade de grupamentos prostéticos com potencial de oxi-redução sucessivamente maiores (figura I.4.) (Voet & Voet, 1995; Di Donato, 2000).
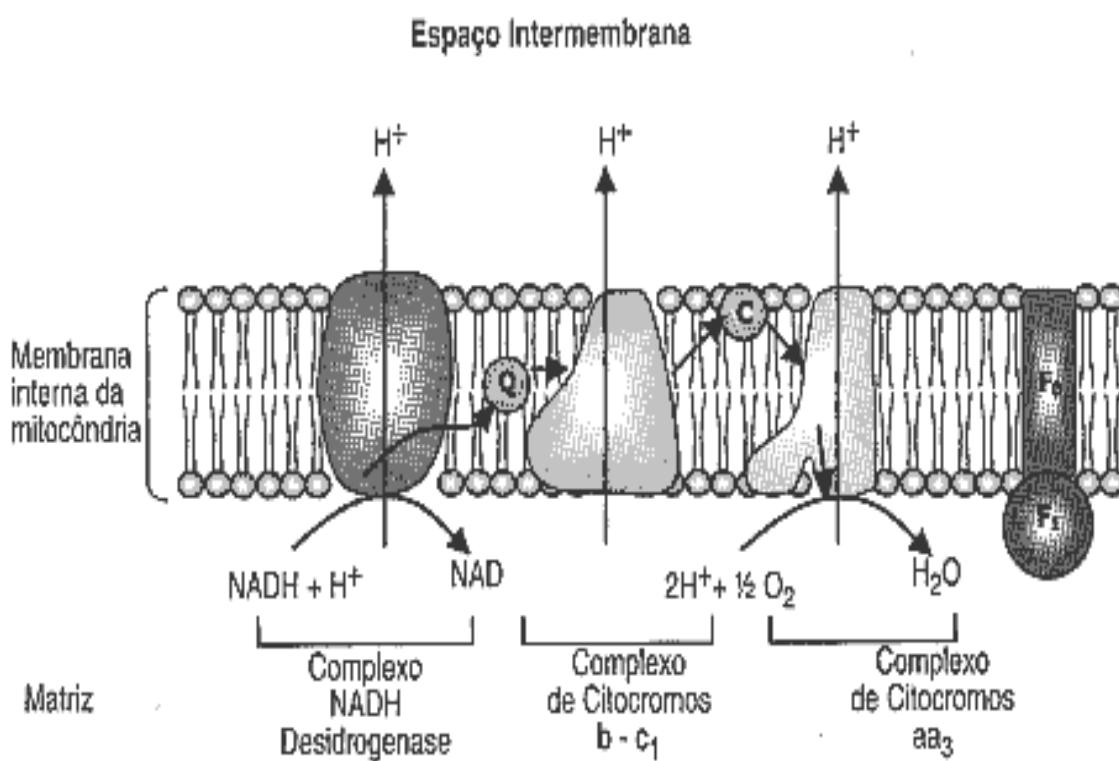
Figura I.4. Cadeia respiratória e fosforilação oxidativa. Observa-se o acoplamento entre o transporte de elétrons entre os complexos enzimáticos e a geração do gradiente de prótons através da membrana mitocondrial interna.

I. 3. 4. 1. NADH – Coenzima Q redutase (complexo I)

O complexo I transfere os elétrons do NADH, derivado da oxidação de ácidos graxos, piruvato e aminoácidos, para a coenzima Q, também chamada ubiquinona (CoQ). É, provavelmente, o maior componente protéico presente na membrana mitocondrial

interna, sendo formado por sete subunidades codificadas pelo DNA mitocondrial e pelo menos 34 subunidades codificadas pelo DNA nuclear (Voet & Voet, 1995; Di Donato, 2000). Com aproximadamente 850kD, o complexo I contém uma molécula de flavina mononucleotídeo (FMN) como grupamento prostético e seis a sete centros ferro-enxofre que participam do processo de transferência de elétrons. FMN e CoQ, as coenzimas do complexo I, podem admitir três estados de oxidação cada. Embora o NADH possa participar com a transferência de dois elétrons, ambos FMN e CoQ são capazes de aceitar e doar um ou dois elétrons por vez, porque suas formas semiquinona são estáveis. Dessa forma, FMN e CoQ são capazes de conduzir elétrons entre um doador capaz de transferir apenas dois elétrons simultaneamente, o NADH, para aceptores capazes de receber um único elétron, os citocromos presentes no complexo III (Voet & Voet, 1995).

I. 3. 4. 2.   Succinato – Coenzima Q redutase (complexo II)

O complexo II é composto por quatro subunidades, todas codificadas pelo DNA nuclear: a enzima dimérica succinato desidrogenase, componente do ciclo do ácido cítrico e três outras pequenas subunidades hidrofóbicas. Esse complexo transfere os elétrons do succinato para a CoQ. Esse processo envolve a participação de um FAD covalentemente ligado, dois centros ferro-enxofre e um citocromo b_{560} (Voet & Voet, 1995; Di Donato, 2000).

I. 3. 4. 3.   Coenzima Q – Citocromo c Redutase (complexo III)

O complexo III passa os elétrons da CoQ reduzida para o citocromo *c*. O complexo III está arranjado assimetricamente na membrana mitocondrial interna e contém 11 subunidades, mas somente três delas apresentam centros redox que são utilizados na conservação de energia. Das três subunidades chaves, o citocromo b é codificado pelo genoma mitocondrial, enquanto o centro ferro-enxofre e o citocromo c_1, bem como todas as oito pequenas subunidades, são codificadas pelo genoma nuclear (Saraste, 1999). O citocromo c_1 está localizado na superfície externa da membrana, enquanto o citocromo *b* é uma proteína transmembrana. O citocromo *b* é particularmente interessante porque contém ambos citocromos tipo *b*, b_H e b_L associados a uma única cadeia polipeptídica (Voet & Voet, 1995).

I. 3. 4. 4. Citocromo c Oxidase (complexo IV)

A citocromo *c* oxidase (COX) é o complexo terminal da cadeia respiratória mitocondrial. A COX transfere os elétrons a partir do ferrocitocromo *c* para o oxigênio molecular, o aceptor final de elétrons. Essa reação está acoplada à transferência de prótons da matriz mitocondrial para o espaço intermembrana, contribuindo para o estoque de energia na forma de gradiente eletroquímico utilizada para a síntese de ATP. A COX consiste de doze ou mais subunidades polipeptídicas (Barrientos *et al*, 2002). As três maiores subunidades formam o centro catalítico da enzima e são codificados pelo DNA mitocondrial. A subunidade I contém os grupamentos heme e um dos íons Cu (Cu_B) enquanto a subunidade II contém um centro de Cu binuclear (Cu_A) (Capaldi, 1992). A subunidade III não apresenta grupamento prostético e não parece estar envolvida no bombeamento de prótons, mas na manutenção da estabilidade estrutural. As demais subunidades, todos codificadas pelo DNA nuclear, são sintetizadas nos ribossomas citoplasmáticos e endereçadas à mitocôndria por meio de diferentes vias de transporte. As funções dessas subunidades ainda não estão bem esclarecidas, mas sua ausência em COX bacterianas sugere que elas não sejam essenciais ao mecanismo catalítico básico de redução do oxigênio e transferência vetorial de prótons (Saraste, 1990; Barrientos *et al*, 2002). A COX é a enzima chave na regulação da produção de energia mitocondrial, uma vez que reação redox entre o citocromo *c* e o oxigênio molecular é essencialmente irreversível (Poyton & McEwen, 1996). Além disso, foi recentemente descoberto um mecanismo de controle da respiração celular baseado na inibição alostérica da COX por elevadas razões de ATP/ADP intramitocondriais (Kadenbach & Arnold, 1999).

I. 3. 4. 5. Síntese de ATP

A síntese de ATP a partir de ADP é realizada pelo complexo V ou ATP sintase. A ATP sintase é composta por duas subunidades codificadas pelo DNA mitocondrial (ATPase 6 e 8) e pelo menos 12 subunidades codificadas pelo DNA nuclear. Funcionalmente e estruturalmente o complexo V é formado por um componente catalítico solúvel na matriz mitocondrial (F_1-ATPase) e um componente de membrana hidrofóbico (F_o-ATPase) que contém o canal de prótons (Saraste, 1999).

O gradiente eletroquímico gerado na CR durante a transferência de elétrons para o oxigênio molecular cria uma polarização na membrana mitocondrial interna, que pode ser revertida pelo fluxo de prótons através do canal de prótons presente no componente F_o da ATP sintase. Esse fluxo de prótons leva à condensação do ADP e fosfato inorgânico em

ATP (Saraste, 1999; Wallace, 1999). A ATP sintase é uma enzima funcionalmente reversível, uma vez que pode catalisar, não apenas a síntese de ATP, usando a força próton motriz através da membrana mitocondrial interna, como também hidrolisa ATP (Saraste, 1999).

I. 3. 5. *Creatina quinase*

As creatina quinases (CK, ATP:creatine *N*-phosphoryltransferase, EC 2.7.3.2) são uma família de enzimas envolvidas que catalisam a transferência reversível de um grupamento N-fosforil de entre fosfocreatina (PCr) e ADP, conforme a reação abaixo (Bittl & Ingwall, 1985; Bessman, 1985).

$$\text{Fosfocreatina} + \text{Mg}^{2+}\text{ADP} + \text{H}^{+} \rightleftharpoons \text{Creatina} + \text{Mg}^{2+}\text{ATP}$$
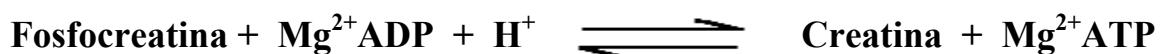
As CKs têm papel fundamental na transferência de energia nas células que apresentam elevado metabolismo energético, fornecendo um sistema eficaz de tamponamento de ATP a esses tecidos (Bessman & Carpenter, 1985). A velocidade de reação da CK excede, em magnitude, a velocidade de síntese de ATP celular. Esse fenômeno pode explicar a habilidade que tecidos excitáveis, como o tecido cardíaco, o músculo esquelético e os neurônios têm para alterar a velocidade de consumo de energia durante os períodos de maior atividade (Bittl & Ingwall, 1985; Saks *et al*, 1996).

As CKs constituem um grupo de diferentes isoformas oligoméricas, com localização subcelular específica. Nos vertebrados existem vários isotipos de CK os quais são produzidos por genes distintos cuja expressão é tecido-específica.

As isoenzimas citosólicas existem exclusivamente como moléculas diméricas, compostas por dois tipos de subunidades, originando três diferentes isoformas: CK-MM e CK-BB, como homodímeros, e o heterodímero CK-MB. A CK-MM é predominantemente encontrada no tecido muscular esquelético maduro e no miocárdio de mamíferos, a CK-BB está presente no cérebro e tecido nervoso periférico, músculo esquelético e cardíaco embrionário de mamíferos, e o heterodímero CK-MB é encontrado somente no tecido cardíaco (Wallimann *et al*, 1992; O'Gorman *et al*, 1996; Hornemann *et al*, 2000).

Estudos cinéticos apontam várias diferenças entre as três isoformas citosólicas, purificadas a partir de tecidos de galinha. Em geral, a isoenzima cerebral (Wallimann *et al*, 1994) mostrou valores maiores de V_{max} e menores K_m para os quatro substratos (ATP, ADP, Cr e PCr). Esse fato permite que a enzima trabalhe de forma mais eficiente mesmo

quando existem baixas concentrações de substrato. Em comparação, a isoforma muscular mostrou uma menor afinidade pelos substratos (K_m maiores) e também menor valor de V_{max}. Isso poderia ser uma adaptação às concentrações relativamente maiores de fosfocreatina (PCr) e creatina (Cr) presentes no tecido muscular esquelético (Wallimann *et al*, 1984; Ventura-Clapier & Veksler, 1994). Por outro lado, os valores para o heterodímero CK-MB não são intermediários entre as duas formas homodiméricas, mas aproximam-se mais da isoforma CK-BB (Hornemann *et al*, 2000). Sua alta afinidade pelos substratos poderia ajudar a garantir uma função apropriada do músculo cardíaco adulto.

As isoenzimas citosólica (Cy-CK) e mitocondrial (Mi-CK) são co-expressas na maioria dos tecidos que possuem CK. Elevada atividade enzimática e níveis altos de mRNA para a Mi-CK estão presentes nos tecidos com elevada demanda energética, como coração, músculo esquelético, cérebro, retina e espermatozóides. Por outro lado, pequenas quantidades são observadas em tecidos contendo músculo liso como útero, placenta e intestino. A Mi-CK sarcomérica (sMi-CK) é quase exclusivamente expressa no coração e músculo esquelético, enquanto a Mi-CK ubíqua (uMi-CK) é principalmente encontrada nos rins, placenta, intestino e cérebro. Dessa forma, parece que a sMI-CK acompanha a CK-M enquanto a uMi-CK acompanha a CK-B (Wyss *et al*, 1992).

Diferentemente das isoformas citosólicas que são estritamente diméricas, as isoenzimas mitocondriais podem ser diméricas ou octaméricas. Embora a Mi-CK seja preferencialmente isolada no estado octamérico, dímero e octâmero são prontamente intercambiáveis. Um equilíbrio dinâmico, dependente de vários parâmetros como pH, concentração de Mi-CK, concentração de substrato e temperatura, tem sido encontrado *in vitro* entre essas formas. As Mi-CKs estão localizadas no espaço intermembranas mitocondrial (Jacobs *et al*, 1964) onde os octâmeros ligam-se à membrana mitocondrial externa, interagindo funcionalmente e, possivelmente, estruturalmente com duas proteínas transmembrana: ao translocador de nucleotídeos de adenina (ATN) na membrana mitocondrial interna e à porina da membrana externa (Eppenberger *et al*, 1967; Brooks & Suelter, 1987; Schlegel *et al*, 1988; Wyss *et al*, 1992; Schlattner *et al*, 1998). As Mi-CK tem acesso preferencial ao ATP gerado a partir da fosforilação oxidativa e exportado da matriz mitocondrial pelo ATN (Saks *et al*, 1985; O'Gorman *et al*, 1996). Regiões enriquecidas em Mi-CK, ATN e porina são chamadas sítios de contato (CS) entre membrana mitocondrial interna e externa (Beutner *et al*, 1996; Beutner *et al*, 1998). Embora as atividades específicas do octâmero e do dímero sejam muito similares, estudos recentes têm demonstrado que *in vivo* o estado octamérico é o funcional. Somente os

octâmeros têm a capacidade de ligar-se as duas membranas mitocondriais opostas e formar os CS (Wallimann *et al*,1992; Wyss *et al*,1992). A desestabilização do octâmero prejudica as suas funções (Khuchua *et al*, 1998; Schlattner & Wallimann, 2000).

Estudos cinéticos realizados a partir de Mi-CK purificadas demonstraram que essa isoforma apresenta menores valores de K_m para MgADP do que para MgATP e menores valores de K_m para PCr do que para Cr (Wyss *et al*, 1992). Semelhante relação tem sido descrita para CK citosólica (Brdiczka *et al*, 1986). A uMi-CK apresenta menor valor de K_m que a sMi-CK, provavelmente refletindo uma adaptação metabólica a baixa concentração de PCr cerebral, quando comparada com os tecidos muscular esquelético e cardíaco. Além disso, para isoenzimas humanas, estudos demonstraram que os substratos PCr e ADP ligam-se mais fortemente às isoformas mitocondriais do que às citoplasmáticas (Kanemitsu *et al*, 1982; Stein *et al*, 1982; Schneider *et al*, 1988).

Embora as isoenzimas CK citosólicas e mitocondriais representem uma classe de proteínas altamente conservadas quanto à seqüência dos aminoácidos, observou-se seis blocos com homologia muito similar separadas por regiões menos conservadas. As regiões mais conservadas estão provavelmente envolvidas em funções essenciais da enzima como atividade catalítica e formação de dímeros. Já as regiões menos conservadas das CKs são provavelmente responsáveis por propriedades específicas, como a formação de octâmeros, ligação a membranas celulares e a miofibrilas (Wyss *et al*, 1992).

As funções propostas para as CKs incluem tamponamento energético, regulação da fosforilação oxidativa e do transporte de potencial químico, na forma de PCr entre os sítios de síntese e utilização de ATP (Brdiczka & Wallimann, 1994; Saks *et al*, 1996). Nesse sentido, parte da atividade das isoformas citosólicas está associada com ATPases em compartimentos subcelulares, tais como retículo sarcoplasmático, ou banda-M miofibrilar, onde a CK está funcionalmente acoplada ao bombeamento de Ca^{+2} (Rossi *et al*, 1990; Korge *et al*, 1993) e a acto-miosina ATPase (Wallimann & Eppenberger, 1985; Krause & Jacobus, 1992), respectivamente.

A inter-relação funcional entre as isoformas mitocondriais e citosólicas é importante na regulação da homeostase energética celular. O ATP produzido a partir da fosforilação oxidativa é convertido em PCr e ADP pela Mi-CK. A PCr formada é transportada ao citosol via porina e o ADP liberado é recaptado para o interior da mitocôndria via ATN, estimulando a fosforilação oxidativa. A enzima citosólica então, utiliza a PCr e re-fosforila o ADP livre localmente disponível, regenerando o *pool* de ATP celular nos sítios de elevado consumo de energia e aumentando a concentração global de

creatina (Bessman & Geiger, 1981; Wallimann *et al*, 1984; Wallimann *et al*, 1985; Wallimann *et al*, 1992; Wyss *et al*, 1992; Schlattner *et al*, 1998). As enzimas CKs participam da regeneração do ATP em associação com ATPases específicas (Friedman & Perryman, 1991). A CK-BB é encontrada em regiões de membrana ricas em receptores colinérgicos, onde pode estar funcionalmente acoplada a Na^+,K^+-ATPase. Assim, a CK-BB pode regular os níveis de ATP necessários ao transporte ativo de íons através da membrana das células cerebrais. Publicações recentes têm sugerido o papel da CK na mielogênese e na divisão celular. (Holtzman *et al*, 1993).

Dois dos quatro isotipos de CK estão expressos no cérebro: o homodímero citoplasmático CK-BB e a isoenzima mitocondrial uMtCK. Foi observado que há maior expressão de CK-BB nos astrócitos e oligodendrócitos do que nos neurônios (Molloy *et al*, 1992).

Devido ao papel central do sistema PCr/CK na regulação da concentração de ATP cerebral, alterações na CK tem sido propostas em doenças neurodegenerativas nas quais ocorre alteração no metabolismo energético. Redução na atividade da CK total tem sido observada em pacientes com Doença de Alzheimer e Doença de Pick (Burbaeva *et al*, 1992; Aksenov *et al*, 1997), sendo a isoforma CK-BB mais afetada que a uMtCK.. Também uma leve diminuição na atividade da CK foi observada no cérebro de pacientes com epilepsia, esquizofrenia e psicose maníaco-depresiva (Burbaeva *et al*, 1987; Burbaeva *et al*, 1990). Essa redução na atividade da CK pode refletir um distúrbio no metabolismo energético cerebral nessas doenças. Além disso, devido a sua importante participação nos processos de manutenção de energia necessária para a mielogênese nos oligodendrócitos, para transporte iônico, de neurotransmissores e mediadores entre astrócitos e neurônios, danos à sua função podem levar a distúrbios na transdução de sinal cerebral (Wallimann *et al*, 1985; Kuzhikandathil & Molly, 1994; Roth *et al*, 1995; Zilles *et al*, 1995).

Diminuição na atividade da CK miocárdica tem também sido implicada na patogênese de cardiomiopatias e falência cardíaca (Saks *et al*, 1991; Veksler & Ventura-Clapier, 1994). A inibição da Mi-CK altera significativamente a regulação da respiração mitocondrial. Essa inibição leva a uma diminuição da sensibilidade da respiração mitocondrial ao ADP. Em células vivas, onde o ADP está presente em concentrações submáximas, essa diminuição da sensibilidade ao ADP pode levar a uma diminuição substancial na produção de energia e velocidade de respiração celular (Kaasik *et al*, 1999).

I. 3. 6. Na^+, K^+ - ATPase

A Na^+,K^+-ATPase é uma enzima integral de membrana encontrada nas células de todos os eucariotos superiores. Essa enzima transloca Na^+ e K^+, contra seus gradientes de concentração, através da membrana plasmática, utilizando energia proveniente de hidrólise do ATP intracelular em ADP como força motriz. A estequiometria de reação pode ser representada da seguinte forma:

$$3\ Na^+\ (intracel.)\ +\ 2\ K^+\ (extracel.)\ +\ ATP\ +\ H_2O \rightleftharpoons$$

$$3\ Na^+\ (extracel.)\ +\ 2\ K^+\ (intracel.)\ +\ ADP\ +Pi$$
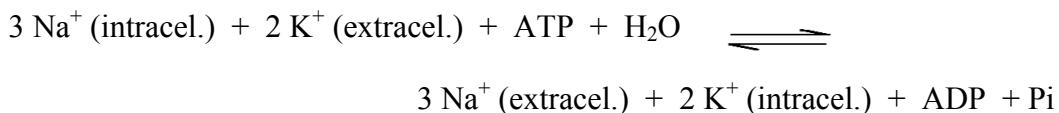
Três cargas positivas (Na^+) são transportadas para o meio extracelular enquanto apenas duas (K^+) são transportadas para o meio intracelular. Dessa forma, o trabalho realizado caracteriza-se como um antiporte eletrogênico, ou seja, o fluxo dos íons Na^+ e K^+ produz um gradiente eletroquímico através da membrana celular (Voet & Voet, 1995).

Esse gradiente é usado como fonte de energia para a formação, despolarização e repolarização do potencial de membrana, para a manutenção e regulação do volume celular, do transporte ativo dependente de Na^+, de glicose, aminoácidos e neurotransmissores e para o cotransporte/antiporte de outros íons (Geering, 1990). De fato, todas as células consomem ATP por elas produzido através da Na^+,K^+-ATPase para a manutenção das concentrações citosólicas de Na^+ e K^+, sendo que esse consumo é de 40 a 60% nas células neuronais (Voet & Voet, 1995).

Quanto à estrutura, a Na^+,K^+-ATPase é um tetrâmero constituído por 2 subunidades polipeptídicas (α e β) covalentemente associadas, incorporadas à bicamada lipídica na membrana plasmática. A subunidade α é formada por 1012 aminoácidos, tem aproximadamente 100 kD é responsável pelas propriedades catalíticas da enzima. Nessa subunidade estão localizados: o domínio para a ligação do ATP, o sítio de fosforilação e os aminoácidos essenciais para a ligação dos cátions. No domínio extracelular também se encontra o sítio de ligação para glicosídios cardiotônicos (Lingrel & Kuntzweiler, 1994). A subunidade β contém em torno de 300 aminoácidos e uma massa de 60 kD. Embora não existam sítios catalíticos nessa subunidade, não é possível separá-la da subunidade α sem perda da atividade enzimática (Gerring, 1990; Skou & Esmann, 1992).

A Na^+,K^+-ATPase tem papel fundamental na manutenção e restauração do gradiente de cátions pela membrana celular. Alterações no mecanismo que mantém o

equilíbrio entre o conteúdo de Na^+ e K^+ intra e extracelulares podem ter conseqüências graves para as células e tem sido associadas com despolarização e instabilidade da membrana.

A inibição da atividade dessa enzima tem sido associada a várias patologias neurológicas (Greene, 1983). Diversas investigações têm relacionado alterações na atividade da Na^+,K^+-ATPase a crises epilépticas e convulsões. Rapport e colaboradores (1975) encontraram uma diminuição de 60% na atividade da Na^+,K^+-ATPase em córtex cerebral obtido de pacientes que apresentavam convulsões generalizadas intratáveis. Além disso, a inibição da atividade da enzima está associada à liberação de neurotransmissores em uma variedade de preparações neuronais (Jacobson *et al*, 1986).

I. 3. 7. *Distúrbios do metabolismo mitocondrial*

Doenças humanas causadas por anormalidades no metabolismo mitocondrial têm sido descritas há mais de 35 anos. Elas compreendem defeitos na oxidação de ácidos graxos, em enzimas do ciclo do ácido cítrico e enzimas da cadeia respiratória e do sistema de fosforilação oxidativa (OXPHOS) (Scriver *et al*, 2002). As alterações envolvendo os complexos enzimáticos da cadeia respiratória têm sido o principal foco no estudo das doenças mitocondriais e representam as mais conhecidas deficiências bioquímicas do metabolismo mitocondrial causadoras dessas patologias. Devido à complexidade de fatores que podem afetar as funções da cadeia respiratória e para uma melhor compreensão dos diferentes mecanismos que podem afetar a OXPHOS, podemos dividir essas anormalidades em dois grandes grupos: defeitos de classe I ou primários e defeitos de classe II ou secundários (Schapira, 1997).

Os defeitos primários são assim definidos por serem causados por mutações nos DNA mitocondrial (mDNA) ou DNA nuclear em genes que codifiquem proteínas componentes das subunidades de qualquer dos complexos da CR. As alterações no DNA nuclear podem incluir mutações que afetem o endereçamento de proteínas à mitocôndria (Schapira, 1997).

Deficiências secundárias na OXPHOS devem-se a interação de fatores genéticos e ambientais. Dentre os fatores genéticos estão incluídas anormalidades no mDNA induzidas por defeitos em genes nucleares afetando a transcrição, translação ou replicação do mDNA, defeitos de reparo no mDNA, defeitos na rota de importação de subunidades codificadas pelo DNA nuclear e defeitos na montagem dos complexos enzimáticos da OXPHOS. Por outro lado, existem evidências de que toxinas, tanto de origem exógena

quanto endógena possam prejudicar a função da OXPHOS. Algumas substâncias como isoquinolinas e radicais livres, como óxido nítrico e ânion superóxido, podem ser gerados endogenamente e inibir complexos da cadeia respiratória (Schapira, 1997).

A função essencial da cadeia respiratória em qualquer tipo celular, exceto nos eritrócitos maduros, bem como a complexidade de suas características bioquímicas e genéticas, faz com que pacientes afetados por desordens mitocondriais apresentem uma variedade de manifestações clínicas distintas. Geralmente, os órgãos com maior demanda energética e que, portanto, são mais dependentes do metabolismo aeróbio, tais como cérebro, músculo esquelético e coração, são mais severamente afetados nessas desordens. Embora originalmente reportadas como miopatias mitocondriais, as manifestações clínicas das mitocondriopatias ocorrem predominantemente como miopatias, encefalopatias e cardiomiopatias (Di Donato, 2000). Similarmente, o rápido aumento no requerimento energético nas fases de crescimento e desenvolvimento tornam os neonatos especialmente vulneráveis as manifestações decorrentes de defeitos no metabolismo energético. Em geral, o quadro clínico neonatal inclui manifestações como hipotonia, letargia, dificuldades alimentar e respiratória, dificuldade de locomoção, retardo psicomotor, convulsões e vômitos. Sem tratamento, a maior parte das crianças morre nos primeiros dias ou meses de vida (Sue *et al*, 1999).

Sintomas neurológicos e neuromusculares são, portanto, as manifestações clínicas mais freqüentes nas desordens de cadeia respiratória. Os efeitos deletérios dos defeitos de OXPHOS parecem ser conseqüência de dois mecanismos principais e provavelmente cooperativos. Primeiro, uma diminuição na produção de energia leva à despolarição neuronal, o que permite a ativação de receptores de aminoácidos excitatórios e prejudica o tamponamento do cálcio intracelular, causando a ativação de proteases e morte celular. Segundo, defeitos na função da cadeira respiratória podem aumentar a produção de espécies reativas de oxigênio, os quais podem danificar as membranas e DNA celular (Beal, 1996). O estudo do metabolismo mitocondrial tem recentemente incluído doenças neurodegerenativas como a doença de Parkinson, a doença de Huntington e a doença de Alzheimer. Defeitos na OXPHOS foram descritos em todas essas patologias. Embora sua relação com a etiologia dessas doenças permaneça ainda indefinida, parece que pelo menos na doença de Parkinson essas alterações contribuem para a morte das células neuronais (Schapira, 1999).

Entretanto, qualquer órgão ou tecido pode teoricamente ser afetado em decorrência de alterações de cadeira respiratória, incluindo trato gastrintestinal e sistema endócrino (Zeviani & Antozzi, 1997).

II.   OBJETIVOS

II. 1. Objetivo geral

Dados recentes da literatura têm mostrado que muitas desordens neuromusculares e neurodegenerativas estão intimamente relacionadas com prejuízo na produção energética pelas mitocôndrias. Portanto, considerando que a etiopatogenia da disfunção neurológica dos pacientes com acidúrias D-2-hidroxiglutárica e L-2-hidroxiglutárica é praticamente desconhecida, o objetivo geral desse estudo foi investigar a influência dos ácidos D-2-hidróxiglutárico (DGA) e L-2-hidróxiglutárico (LGA) sobre diversos parâmetros importantes do metabolismo energético celular, na esperança de identificar possíveis alterações que poderiam explicar a fisiopatologia do dano tecidual nessas desordens.

II. 2. Objetivos específicos

- Verificar o efeito *in vitro* dos DGA e LGA sobre a utilização de glicose em córtex cerebral de ratos jovens.
- Verificar o efeito *in vitro* dos DGA e LGA sobre a produção de CO_2 em córtex cerebral de ratos jovens.
- Verificar o efeito *in vitro* dos DGA e LGA sobre os complexos enzimáticos da cadeia respiratória de córtex cerebral, músculo esquelético e músculo cardíaco de ratos jovens.
- Verificar o efeito *in vitro* dos DGA e LGA sobre a atividade da Na^+, K^+-ATPase da membrana plasmáticas sináptica de córtex cerebral e cerebelo de ratos jovens.
- Verificar o efeito *in vitro* dos DGA e LGA sobre a atividade da creatina quinase em homogeneizado total e nas frações citosólica e mitocondrial de córtex cerebral, cerebelo, músculo cardíaco e músculo esquelético de ratos jovens.

III. MATERIAL, MÉTODOS E RESULTADOS

III. 1. Inibição da atividade da citocromo c oxidase em córtex cerebral de ratos e músculo esquelético humano pelo ácido D-2-hidroxiglutárico *in vitro*.

Inhibition of cytochrome *c* oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid *in vitro*

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Inhibition of cytochrome *c* oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid in vitro

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Abstract

L-2-Hydroxyglutaric (LGA) and D-2-hydroxyglutaric (DGA) acids are the characteristic metabolites accumulating in the neurometabolic disorders known as L-2-hydroxyglutaric aciduria and D-2-hydroxyglutaric aciduria, respectively. Although these disorders are predominantly characterized by severe neurological symptoms, the neurotoxic mechanisms of brain damage are virtually unknown. In this study we have evaluated the role of LGA and DGA at concentrations ranging from 0.01 to 5.0 mM on various parameters of energy metabolism in cerebral cortex slices and homogenates of 30-day-old Wistar rats, namely glucose uptake, CO_2 production and the respiratory chain enzyme activities of complexes I to IV. DGA significantly decreased glucose utilization (2.5 and 5.0 mM) by brain homogenates and CO_2 production (5 mM) by brain homogenates and slices, whereas LGA had no effect on either measurement. Furthermore, DGA significantly inhibited cytochrome *c* oxidase activity (complex IV) (EC 1.9.3.1) in a dose-dependent manner (35–95%) at doses as low as 0.5 mM, without compromising the other respiratory chain enzyme activities. In contrast, LGA did not interfere with these activities. Our results suggest that the strong inhibition of cytochrome *c* oxidase activity by increased levels of DGA could be related to the neurodegeneration of patients affected by D-2-hydroxyglutaric aciduria. © 2002 Published by Elsevier Science B.V.

Keywords: L-2-Hydroxyglutaric acid; D-2-Hydroxyglutaric acid; Brain metabolism; Cytochrome *c* oxidase

1. Introduction

L-2-Hydroxyglutaric aciduria (LHGA) and D-2-hydroxyglutaric aciduria (DHGA) are rare neurometabolic disorders biochemically characterized by tissue accumulation and high urinary excretion of L-2-hydroxyglutaric acid (LGA) and D-2-hydroxyglutaric acid (DGA), respectively [1–6].

LHGA was first described in 1980 by Duran and colleagues [2] and has now been described in at least 50 patients. Patients with LHGA usually present

with mental deterioration, seizures, pyramidal and extrapyramidal symptoms and severe cerebellar dysfunction. Ataxia, tremor and choreiform movements, as well as speech delay in infancy or childhood have also been reported [4,7,8]. Although in most patients symptoms presentation occurs in early to middle childhood, a more severe form has been reported in an infant which presented with hypotonia, apnoea and convulsions shortly after birth [9]. An adult form affecting older patients has also been reported [10]. Neuroimaging reveals cerebellar atrophy, progressive loss of myelinated arcuate fibres and alterations in the basal ganglia. Besides the characteristic high levels of LGA in plasma, CSF and urine, a few affected patients present increased concentrations of lactate or Krebs cycle intermediates in CSF, plasma or urine [4,9,11].

DHGA was first recognized by Chalmers and co-workers in 1980, and has now been reported in at least 25 patients [1,6]. It is clinically characterized by at least two variants, a severe form with early-infantile-onset encephalopathy, whose patients usually present with seizures, hypotonia and poor development, as well as enlarged frontal subarachnoid spaces and subdural effusions and signs of cerebral delayed maturation with subependymal cysts in the caudate nucleus detected by MRI. The other variant is clinically milder and more variable, presenting usually with mental retardation, macrocephaly and hypotonia. Delayed cerebral maturation, ventriculomegaly and subependymal cysts are also observed by MRI. Biochemically, besides the high excretion of DGA, lactate and Krebs cycle intermediates are also found in elevated amounts in the urine of a considerable number of patients [5,6]. High excretion of DGA also occurs in multiple acyl-CoA dehydrogenase deficiency (glutaric acidemia type II), which is due to a defect of the electron transfer flavoprotein (ETP) or of the mitochondrial enzyme ETF-ubiquinone oxido-reductase [12].

Despite the intensive clinical investigation and the large number of loading and fast tests, the underlying biochemical defect of these disorders and the origin of DGA and LGA have remained an enigma. Initially L-2-hydroxyglutaric acid dehydrogenase and D-2-hydroxyglutaric acid dehydrogenase deficiencies were considered as potential causes of these diseases. However, the activities of the enzymes were normal

or even increased in the liver of affected patients, suggesting that their accumulation may result from a secondary pathway, rather than from the primary substrate of the missing enzyme activity [3,4,13]. Likewise, the effects of these acids on cell metabolism are virtually unknown and await investigation.

Moreover, virtually nothing is known about the pathophysiology of the neurological dysfunction of DHGA and LHGA. However, considering that elevated levels of lactate and/or Krebs cycle intermediates are observed in some patients affected by these disorders, it is conceivable that a primary or functional mitochondrial defect or dysfunction may be associated with these diseases. Therefore, in the present study we investigated the in vitro effects of DGA and LGA on various parameters of energy metabolism such as glucose utilization and CO_2 formation and on the enzyme activities of the respiratory chain complexes in cerebral cortex of young rats in the hope to determine whether the acids could compromise energy production in the brain. Skeletal muscle from human beings were also used in some experiments since, similarly to brain, this tissue has a high amount of mitochondria and thus a high demand of energy.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for the radiolabeled compounds [U-^{14}C]acetate and [U-^{14}C]citrate which were purchased from Amersham International plc, UK.

2.2. Subjects

Thirty-day-old Wistar rats bred in our laboratory were used. Rats had free access to a 20% protein commercial chow and water and were kept in a room with a 12:12-h light/dark cycle and temperature of $24 \pm 1°C$. Animals were killed by decapitation without anesthesia, the brains were immediately removed and the cerebral cortex was dissected onto an ice-cold glass plate. The experimental protocol was approved by the Ethics Committee for animal re-

search of the Federal University of Rio Grande do Sul, Porto Alegre.

Skeletal muscle specimens obtained from normal human individuals were also used for cytochrome *c* oxidase activity determination. All subjects gave written informed consent to participate in the study. The research was approved by the Committee for Human Research of Hospital de Clínicas de Porto Alegre.

2.3. Tissue preparation

2.3.1. Cerebral cortex slice preparation

Brain cortex was cut to produce 400-μm wide slices using a McIlwain chopper. Slices were pooled, weighed and used for the glucose uptake and CO_2 production assays.

2.3.2. Homogenate preparation

Rat cerebral cortex was homogenized (1:10, w/v) in Krebs–Ringer bicarbonate buffer, pH 7.0 (glucose uptake) or pH 7.4 (CO_2 production), or in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 UI ml^{-1} heparin) (respiratory chain enzyme activities). The homogenates were centrifuged at $800 \times g$ for 10 min and the supernatants kept at $-70°C$ until used for enzyme activity determination. The maximal period between homogenate preparation and enzyme analysis was always less than 5 days.

Human skeletal muscle was homogenized in the same way and the supernatants were used for the determination of cytochrome *c* oxidase (COX) activity. The protein concentration in the supernatants (brain and skeletal muscle) varied from 1.0 to 2.0 mg protein ml^{-1}.

2.4. Glucose utilization

Cerebral cortex slices (100 mg) or a corresponding volume of homogenates (1:10, w/v) prepared in Krebs–Ringer bicarbonate buffer, pH 7.0 (KRB) from overnight-fasted animals were incubated in the same buffer (in a total volume of 1 ml), containing 5.0 mM glucose and DGA or LGA (1–5 mM) in a O_2/CO_2 (19:1) mixture in a metabolic shaker at 37°C for 60 min. Flasks were firstly pre-incubated for 15 min (90 oscillations min^{-1}) in the absence of

glucose. Glucose was then added at the beginning of incubation. Control experiments did not contain the acids in the incubation medium [14]. Glucose was measured by the glucose oxidase method [15] and the uptake determined by subtracting the amount after incubation from the total amount measured before incubation.

2.5. CO_2 production

Cerebral cortex slices (50 mg) or a corresponding volume of homogenates (1:10, w/v) were added to small flasks (11 cm^3) containing 0.5 ml Krebs–Ringer bicarbonate buffer, pH 7.4. Flasks were pre-incubated in a metabolic shaker at 37°C for 15 min (90 oscillations min^{-1}). After pre-incubation, 0.2 μCi [U-^{14}C]acetate and 0.5 mM of the unlabeled acetate were added to the incubation medium. In some experiments, 0.1 μCi [U-^{14}C]citrate and 0.5 mM of the unlabeled substrate were used. DGA or LGA (buffered to pH 7.4) was added to the incubation medium at final concentrations of 1.0, 2.5 or 5.0 mM. The controls did not contain the acids. The flasks were gassed with a O_2/CO_2 (95:5) mixture and sealed with rubber stoppers and Parafilm M. Glass center wells containing a folded 65 mm/5 mm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min of incubation at 37°C, 0.1 ml of 50% trichloroacetic acid was added to the medium and 0.1 ml of benzethonium hydroxide was added to the center wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete CO_2 trapping and then opened. The filter papers were removed and added to vials containing scintillation fluid, and radioactivity was measured [16].

2.6. Respiratory chain enzyme activities

The activities of citrate synthase (EC 4.1.3.7) and of the respiratory chain enzyme complexes succinate-DCIP-oxidoreductase (complex II) (EC 1.3.5.1) and succinate:cytochrome *c* oxidoreductase (complex II+CoQ+complex III) (EC 1.10.2.2) were determined in brain cortex homogenates according to the method of Fischer et al. [17]. The activity of cytochrome *c* oxidase (complex IV) (COX) (EC 1.9.3.1) was measured according to Rustin et al. [18], whereas those

of NADH:cytochrome *c* oxidoreductase (complex I+CoQ+complex III) (EC 1.6.5.3) and ubiquinol cytochrome *c* oxidoreductase (complex III) (EC 1.10.2.2) were assayed according to the method described by Schapira et al. [19]. Experimental groups contained various concentrations of LGA or DGA (1, 2.5 and 5 mM). Control groups did not contain any acid in the incubation medium.

2.7. Protein determination

Protein was measured by the method of Lowry et al. [20] using bovine serum albumin as standard.

A

B

Fig. 2. Effect of D-2-hydroxyglutaric acid (DGA) and L-2-hydroxyglutaric acid (LGA) on in vitro CO_2 production from [U-^{14}C]acetate (μmol h^{-1} (g tissue)$^{-1}$) by cerebral cortex homogenates of 30-day-old rats. Values are mean $\pm$ S.E.M. for $n = 4$–7 per group, and are expressed as percentage of controls. *$P < 0.05$, compared to controls (Duncan multiple range test).

2.8. Statistical analysis

Unless otherwise stated, results are presented as means $\pm$ standard error of the mean. Data concerning glucose utilization, CO_2 production and the activities of the respiratory chain enzyme complexes were analyzed by one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when *F* was significant. The Student *t*-test for paired samples was also used for comparison of two means. Differences between the groups were rated significant at a probability error of less than 0.05.

A

B

Fig. 1. Effect of D-2-hydroxyglutaric acid (DGA) on in vitro glucose uptake (μmol h^{-1} (g tissue)$^{-1}$) by cerebral cortex of 30-day-old rats. Values are mean $\pm$ S.E.M. for $n = 4$–7 per group. *$P < 0.05$, **$P < 0.01$ compared to controls (Duncan multiple range test). (A) Slices; (B) homogenates.

3. Results

We first determined the effect of DGA (Fig. 1) and LGA (Fig. 2), at concentrations varying from 1 to 5 mM, on glucose utilization by cerebral cortex slices and homogenates of 30-day-old rats. DGA at concentrations of 2.5 mM and higher significantly reduced the utilization of glucose by the cerebral homogenates ($F_{(3,14)} = 5.5663$; $P = 0.0143$), but not by the cerebral cortex slices ($F_{(3,23)} = 0.2354$; $P = 0.8706$) (Fig. 1). LGA (5 mM) had no effect on this parameter either in slices ($t_{(7)} = 0.45$; $P = 0.671$) or in homogenates ($t_{(7)} = 0.849$; $P = 0.424$) (results not shown).

Fig. 2 shows the in vitro CO_2 production from [U-^{14}C]acetate in rat cerebral cortex homogenates in the presence of 1.0, 2.5 or 5.0 mM DGA (Fig. 2A) or LGA (Fig. 2B). Values are expressed as percentage of controls. CO_2 production ranged from 81 to 156 μmol h^{-1} (mg tissue)$^{-1}$ in the control group. It can be seen that 5 mM DGA significantly reduced CO_2 production in homogenates ($F_{(3,27)} = 3.1537$; $P = 0.0433$), whereas LGA had no effect ($F_{(3,15)} = 0.6739$; $P = 0.5845$). Fig. 3 shows the in vitro CO_2 production from [U-^{14}C]acetate in rat cerebral cortex slices in the presence of 5.0 mM DGA (Fig. 3A) or LGA (Fig. 3B). DGA significantly reduced CO_2
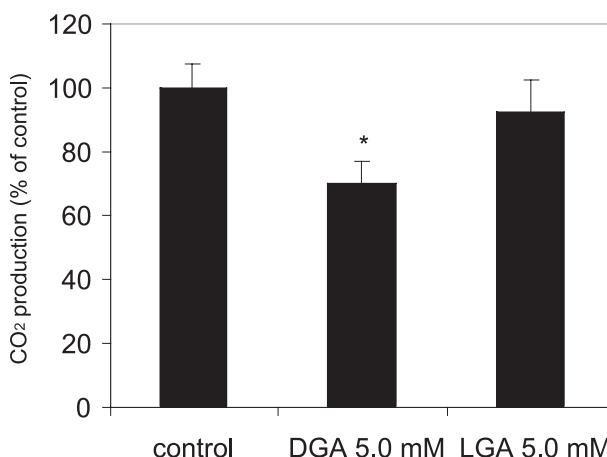
Fig. 4. Effect of D-2-hydroxyglutaric acid (DGA), L-2-hydroxyglutaric acid (LGA) and malonate (MA) on in vitro CO2 production from [U-^{14}C]citrate (μmol h^{-1} (g tissue)$^{-1}$) by cerebral cortex slices of 30-day-old rats. Values are mean $\pm$ S.E.M. for $n = 3$ per group, and are expressed as percentage of controls. *$P < 0.05$, compared to controls (Duncan multiple range test).

production, whereas LGA had no effect ($F_{(2,11)} = 4.4614$; $P = 0.0451$).

We also examined the effect of 5 mM LGA and DGA on the in vitro CO_2 production from [U^{14}C]citrate by cerebral cortex slices (Fig. 4). In these experiments we also used 5 mM malonate (MA), a classical and potent succinate dehydrogenase inhibitor. The results showed that malonate and DGA significantly inhibited CO_2 production, whereas LGA caused no effect ($F_{(3,11)} = 8.1022$; $P = 0.0083$).

Next, we investigated the effect of DGA and LGA on the activities of the respiratory chain enzyme complexes in homogenates of rat brain cortex. DGA strongly reduced the activity of cytochrome c oxidase (COX) ($F_{(7,43)} = 51.1114$; $P = 0.00001$) in a dose-dependent way, whereas the activities of complex I+III ($F_{(3,15)} = 0.2127$; $P = 0.8856$), complex II ($F_{(3,23)} = 0.1130$; $P = 0.9392$), succinate dehydrogenase (SDH) ($F_{(3,23)} = 0.3832$; $P = 0.7662$), complex II+III ($F_{(3,15)} = 2.4241$; $P = 0.1163$), and complex III ($F_{(2,23)} = 0.9773$; $P = 0.4231$) were not affected by the acid (Fig. 5). In contrast, LGA did not alter these activities (complex I+III: $F_{(3,15)} = 0.04435$; $P = 0.7262$; complex II: $F_{(3,23)} = 0.2242$, $P = 0.8784$; SDH: $F_{(3,31)} = 0.499$, $P = 0.985$; complex II+III, $F_{(3,15)} = 0.1529$; $P = 0.9258$; complex III $F_{(3,15)} = 0.3385$; $P = 0.7999$; complex IV: $F_{(3,15)} = 0.190$; $P = 0.9962$) (results not shown).

The concentration of DGA required to inhibit 50%

Fig. 3. Effect of D-2-hydroxyglutaric acid (DGA) and L-2-hydroxyglutaric acid (LGA) on in vitro CO2 production from [U-^{14}C]acetate (μmol h^{-1} (g tissue)$^{-1}$) by cerebral cortex slices of 30-day-old rats. Values are mean $\pm$ S.E.M. for $n = 4$ per group, and are expressed as percentage of controls. *$P < 0.05$, compared to controls (Duncan multiple range test).
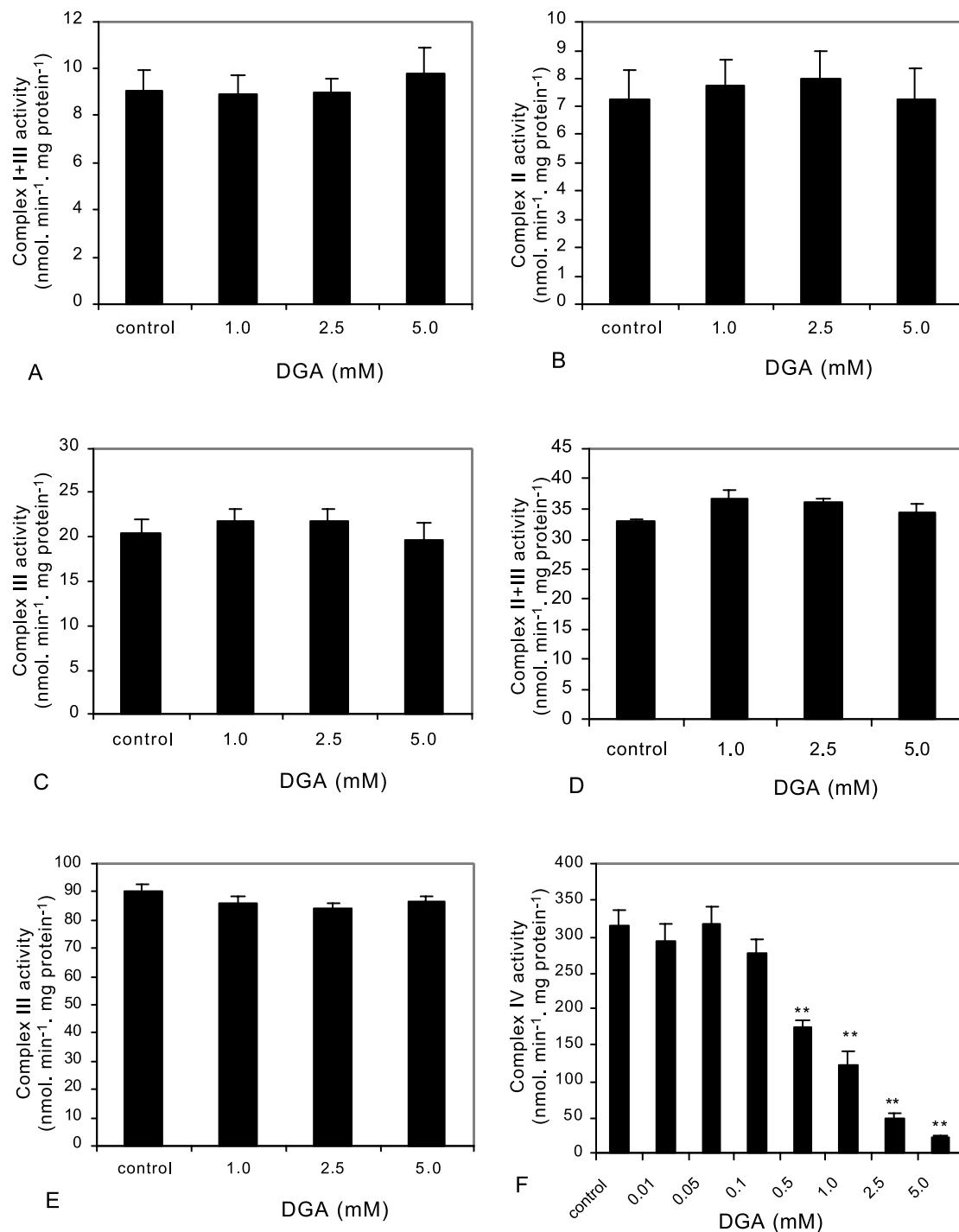
Fig. 5. Effect of D-2-hydroxyglutaric acid (DGA) on the activities (nmol min^{-1} (mg protein)$^{-1}$) of the respiratory chain enzyme complexes in cerebral cortex homogenates of 30-day-old rats. Values are mean ± S.E.M. for $n = 4$–9 independent experiments per group of the respiratory chain complexes activities measured in cerebral cortex homogenates in the presence or absence of the metabolites. **$P < 0.01$ compared to controls (Duncan multiple range test).
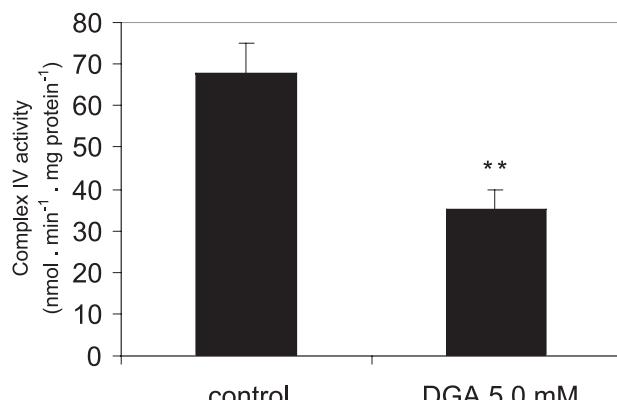
Fig. 6. The effect of D-2-hydroxyglutaric acid (DGA) on cytochrome c oxidase activity (nmol min^{-1} (mg protein)$^{-1}$) from human skeletal muscle. Values are mean ± S.E.M. for $n = 6$ per group. **$P < 0.01$ compared to controls (Student t-test).

of COX activity in cerebral cortex of rats was measured as the IC$_{50}$ [21]. The data indicated an IC$_{50} = 0.314 \pm 0.061$ mM (mean ± S.D.).

In order to test whether the significant reduction of COX activity caused by DGA was due to a non-specific effect of D-enantiomers, we also tested the effect of 5 mM D-2-hydroxy-3-methylvaleric acid (HMVA), a compound with a molecular mass similar to that of DGA on this activity. The acid did not alter COX activity in homogenates of rat cerebral cortex ($t(6) = 1.09$; $P = 0.319$) (results not shown).

The next step was to investigate whether the inhibition of COX activity verified in rat brain also oc-

curs in human tissues. Thus, the effect of 5 mM DGA on COX activity from human skeletal muscle homogenates was examined. We observed that the acid significantly inhibited COX activity in skeletal muscle ($t(10) = 3.786$; $P = 0.004$) (Fig. 6).

The kinetics of the interaction of DGA with COX in homogenates from cerebral cortex was also determined [22]. The Lineweaver–Burk double-reciprocal plot was analysed over a range of cytochrome c concentrations (0.008–0.05 μM) in the absence or presence of DGA (0.5–2.5 mM). The data indicate that the inhibition of COX activity by DGA is uncompetitive (Fig. 7). The K_m calculated was 0.0539 ± 0.011 mM (mean ± S.D., $n = 3$). The K_i value (the dissociation constant of the enzyme–substrate–inhibitor complex) was calculated by the method of Dixon [21], which provides a simple way of determining the inhibition constant (K_i) for uncompetitive inhibitors. The K_i value calculated was 0.226 ± 0.022 mM for DGA (mean ± S.D., $n = 5$).

4. Discussion

High amounts of DGA and LGA accumulate in DHGA and LHGA, respectively. Although severe neurological symptoms and structural brain abnormalities are frequently found in these neurometabolic diseases, very little is known about the pathophysio-
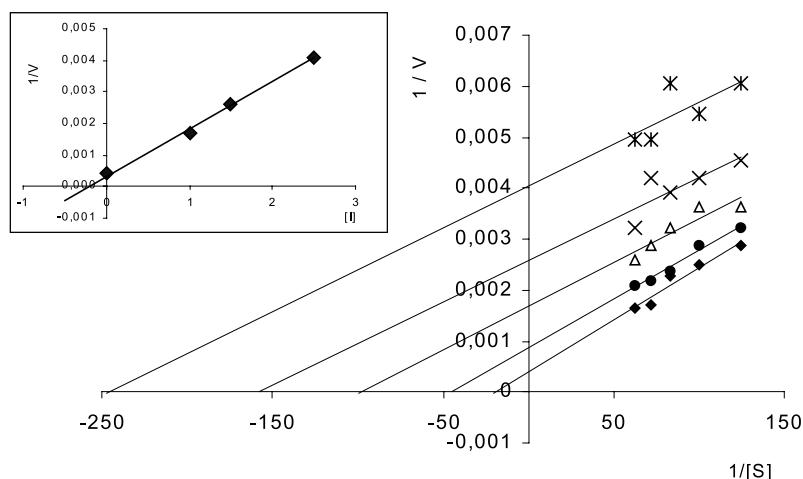
Fig. 7. Kinetic analysis of the inhibition of cytochrome c oxidase from cerebral cortex of rats by D-2-hydroxyglutaric acid. The graph shows a double reciprocal plot of cytochrome c oxidase for cytochrome c concentrations (0.008–0.025 μM) ([S]) in the absence (♦, controls) and in the presence of 0.5 (●), 1.0 (△), 1.5 (×) and 2.5 (*) mM D-2-hydroxyglutaric acid ([I]). The inset shows the Dixon plot for K_i determination. All experiments were repeated at least three times and similar results were obtained. Data presented are representative of one experiment.

logical mechanisms underlying the neurological dysfunction of DHGA and LHGA [5,23]. The understanding of the biochemical alterations in brain may possibly contribute to a better therapeutic management and consequently to higher survival rates for the affected patients.

The present study was undertaken to evaluate the influence of high concentrations of DGA and LGA on some biochemical parameters of energy metabolism in rat brain. We firstly observed that DGA decreased brain glucose utilization in cerebral cortex homogenates, but not in cortical slices, and that LGA had no effect on this parameter. The lower rate of glucose utilization by homogenates may reflect a decreased Krebs cycle activity. Moreover, the lack of effect of DGA with slices may be due to a lower penetration of the metabolite to all of the cells in the slice. In this context, the next set of experiments were designed to investigate the activity of the Krebs cycle by measuring CO_2 production from acetate. A significant reduction of CO_2 production in brain homogenates and slices was detected due to the presence of DGA in the incubation medium, whereas LGA had no effect on this parameter. Although we do not know how DGA crosses cell membranes, a possible competition between DGA and acetate through the same membrane transporter (monocarboxylic carrier) may reduce CO_2 production from [$U^{14}C$]acetate. However, this is unlikely since CO_2 generation from citrate, which uses the tricarboxylic carrier to enter mitochondria, was also blocked by DGA. Thus, the data are indicative that the inhibitory action of DGA is probably secondary to a blockage of the Krebs cycle and/or the respiratory chain.

In this context, we verified that DGA strongly inhibited COX (complex IV) activity in a concentration-dependent manner, whereas the other activities of the respiratory chain were not changed by the metabolite. On the other hand, LGA did not alter the respiratory chain complex activities studied. The results indicate that DGA is an inhibitor of only one enzyme of the electron transfer respiratory chain involved in the oxidative phosphorylation and responsible for most energy produced in the cell. The kinetic analysis of the effects of DGA on COX activity indicated an uncompetitive inhibition. The K_i value obtained for the inhibition of COX by DGA was of

the millimolar order. Therefore, it is likely that suppression of COX activity by DGA is the primary cause of the reduction of both glucose uptake and CO_2 production (Krebs cycle) observed in the present study. Further studies are clearly required to verify the in vivo effect of DGA on COX activity and this investigation is currently in progress in our laboratory.

Furthermore, the observations that LGA did not interfere with the parameters studied and that D-2-hydroxymethylvaleric acid, a D-isomer of molecular mass similar to that of DGA, caused no alteration of COX activity point to a specific effect of DGA, rather than a nonspecific action due to acidic or to D-enantiomer compounds. As regards LGA, our results are in agreement with a previous report demonstrating that the activities of complexes I to IV of the respiratory chain are normal in liver and muscle biopsies from patients with LHGA, indicating that the sustained tissue elevation of LGA does not provoke inhibition of these activities [4].

We also verified in the present study that DGA inhibits COX activities in human skeletal muscle homogenates, and this is not surprising since, due to its critical importance for life support, in general the chemical structure of cytochrome oxidase was well preserved along evolution.

Cytochrome *c* oxidase (COX; EC 1.9.3.1) or complex IV, the last component of the respiratory chain, catalyses the transfer of electrons from cytochrome *c* to molecular oxygen. It consists of 13 subunits, 10 of which are encoded by nuclear DNA [24]. A defect in this complex causes an inability to produce energy aerobically and results in the accumulation of lactic acid. The tissues that are more dependent on aerobic metabolism, such as brain, muscle and heart, are more likely to be affected in these disorders. This is in line with the fact that COX deficiency usually causes an encephalopathy or a myopathy, although the most common clinical presentation is encephalopathy [25–33].

The regulation of the rate of respiration and ATP synthesis was previously thought to be only due to the electrochemical proton gradient across the inner mitochondrial membrane according to the chemiosmotic hypothesis [34]. More recently, another mechanism of respiratory control was attributed to the allosteric inhibition of COX at high intramitochon-

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drial ATP/ADP ratios [35–37]. In a recent report, Kadenbach and colleagues [38] demonstrated a reversible and cAMP-dependent phosphorylation of nuclear-coded subunits of COX at high ATP levels causing its inhibition and hypothesized that this may represent a general mechanism of energy metabolism control based on variable efficiency of energy transduction in COX and on the turning on and off of respiratory control via the intramitochondrial ATP/ADP ratio.

Although neurological symptoms are common in primary mitochondrial disorders, very little is known about the influence of the metabolites accumulating in neurodegenerative disorders on the activities of the respiratory chain complexes. In the present study we demonstrated that D-hydroxyglutaric acid strongly inhibits COX activity in brain of young animals. The degree of this inhibition ranged from 45% to 90% according to the concentration of DGA used in the assays (0.5–5 mM). The question which must be raised is whether this extent of inhibition could compromise energy production. A recent report showed that the control of COX flux is tightly regulated in human skeletal muscle in vivo with important implications for mitochondrial myopathies [39]. These investigators demonstrated that significant reductions of the respiratory fluxes are evident even at a low degree of inhibition of the isolated COX. They observed significant effects on the flux control coefficient and the COX reserve capacity in samples having 10–50% deleted mtDNA, reflected by a decline in COX activity and heme aa3 content, and presumed that these findings might possibly explain the pathological phenotype occurring in individuals carrying a low proportion of mutant mtDNA in susceptible tissues. All this indicates a rate limitation of oxidative phosphorylation by COX. Therefore, the degree of inhibition (45–90%) found in the present study probably leads to blockage of the aerobic glycolytic pathway and to energy deprivation. Therefore, it is tempting to speculate that energy deprivation may be related to the symptomatology and brain damage seen in patients affected by DHGA.

In the present study, significant inhibition of COX activity was achieved with 0.5 mM DGA, which is within the blood circulating (46–757 μmol l^{-1}) levels of DGA in patients affected by DHGA [5,6]. Although the brain concentrations of DGA in these patients are yet unknown, we cannot exclude that higher intracerebral concentrations of DGA may be attained in this neurometabolic disease, as possibly occurs in other organic acidemias so-called 'cerebral' organic acidemias whose symptoms are predominantly or almost exclusively neurologic [40]. Therefore, if these results can be extrapolated to the human condition, it is conceivable that this concentration (0.5 mM) may affect the respiratory chain flux in tissues of these patients.

The molecular defect of DHGA has not been established yet. It has been proposed that D-2-hydroxyglutaric aciduria could be due to a primary mitochondrial defect, with a separate ETF-linked of ETF-ubiquinone oxireductase-linked dehydrogenase being the molecular underlying defect [5]. Although tissue accumulation of DGA is the biochemical hallmark of D-hydroxyglutaric aciduria, some of the documented patients excrete increased amounts of lactate and citric acid cycle intermediates or dicarboxylic acids, pointing to a primary or functional mitochondrial dysfunction [6]. The present findings point to a secondary mitochondrial respiratory chain dysfunction caused by the metabolite which most accumulates in this disorder. In this context, the elevation in the levels of Krebs cycle intermediates found in some of the affected patients may occur secondarily to the blockage of the respiratory chain electron flux leading to an increase of NADH and FADH2 concentrations. Increase of these reduced nucleotides may possibly lead to inhibition of alpha-ketoglutaric acid and succinate dehydrogenases and consequently of their precursors alpha-ketoglutarate and succinate. Lactate may also be increased because of the high NADH/NAD$^+$ ratio. In this context, it would be important to determine whether the DGA levels are more elevated in patients excreting or accumulating these intermediates.

Now concerning to the neuronal degeneration in DHGA, lack of energy might compromise the synthesis of neurotransmitters (acetylcholine, glutamate, aspartate and GABA) from citric acid intermediates [41] and lipid synthesis in brain which could cause serious neurological damage. This may be the case for DHGA where the patients have demyelination and cerebral cortical atrophy [5].

On the other hand, our present findings of impaired mitochondrial energy production in brain

caused by DGA may lead to activation of the NMDA receptors, even at normal concentrations of glutamate in the synaptic cleft, by a reduction of the resting membrane potential, inducing a release of the voltage-dependent Mg^{2+} block of the channel resulting in calcium influx and eventually cell death (slow-onset excitotoxicity) [42]. It should also be stressed that lack of energy may cause a deficient glutamate uptake by glial cells and by neurons, leaving more neurotransmitter in the cleft.

In conclusion, although the biochemical defect of DHGA is still unknown, it is possible that a reduction of COX activity caused by DGA as found in the present study may be related to the neurodegeneration of patients affected by DHGA. Inhibition of this activity could also explain the elevation of some of the Krebs cycle intermediates and lactate in the body fluids of some of these patients. It is also interesting to point out that hypotonia and myocardiopathy, commonly seen in COX deficiency, are frequently observed in DHGA patients with the early severe form. On this basis, it is possible that some of our results may be relevant to the understanding of the biochemical alterations leading to neurologic deterioration in these patients.

Acknowledgements

References

[1] R.A. Chalmers, A.M. Lawson, R.W.E. Watts, A.S. Tavill, J.P. Kamerling, E. Hey, D. Ogilvie, D-2-Hydroxyglutaric aciduria: case report and biochemical studies, J. Inherit. Metab. Dis. 3 (1980) 11–15.

[2] J.P. Duran, H.D. Kamerling, A.H. van Gennip Bakker, S.K. Wadman, L-2-Hydroxyglutaric aciduria: an inborn error of metabolism?, J. Inherit. Metab. Dis. 3 (1980) 109–112.

[3] R.J.A. Wanders, L. Vilarinho, H.P. Hartung, G.F. Hoffmann, P.A.W. Mooijer, G.A. Jansen, J.G.M. Huijmans, J.B.C. de Klerk, H.J. tem Brink, C. Jakobs, M. Duran, L-2-Hydroxyglutaric aciduria: Normal L-2-hydroxyglutarate dehydrogenase activity in liver from two new patients, J. Inherit. Metab. Dis. 20 (1997) 725–726.

[4] P.G. Barth, R.J.A. Wanders, H.R. Scholte, N. Abeling, C. Jakobs, R.B.H. Schutgens, P. Vreken, L-2-Hydroxyglutaric aciduria and lactic acidosis, J. Inherit. Metab. Dis. 21 (1998) 251–254.

[5] M.S. van der Knaap, C. Jakobs, G.F. Hoffmann, W.L. Nyhan, W.O. Renier, J.A.M. Smeitink, C.E. Catsman-Berrevoets, O. Hjalmarson, H. Vallance, K. Sugita, C.M. Bowe, J.T. Herrin, W.J. Craigen, N.R.M. Buist, D.S.K. Brookfield, R.A. Chalmers, D-2-Hydroxyglutaric aciduria: biochemical marker or clinical disease entity?, Ann. Neurol. 45 (1999) 111–119.

[6] M.S. van der Knaap, C. Jakobs, G.F. Hoffman, M. Duran, A.C. Muntau, S. Schweitzer, R.I. Kelley, F. Parrot-Rouland, J. Amiel, P. De Lonlay, D. Rabier, O. Eeg-Olofsson, D-2-Hydroxyglutaric aciduria: further clinical delineation, J. Inherit. Metab. Dis. 22 (1999) 404–413.

[7] P.G. Barth, G.F. Hoffmann, J. Kaeken, W. Lehnert, F. Hanefeld, A.H. van Gennip, M. Duran, J. Valk, R.B. Schutgens, F.K. Trefz et al., L-2-Hydroxyglutaric acidemia: a novel inherited metabolic disease, Ann. Neurol. 32 (1992) 66–71.

[8] P.G. Barth, G.F. Hoffmann, J. Jaeken, R.J. Wanders, M. Duran, G.A. Jansen, C. Jakobs, W. Lehnert, F. Hanefeld, J. Valk et al., L-2-Hydroxyglutaric acidemia: clinical and biochemical findings in 12 patients and preliminary report on L-2-hydroxyacid dehydrogenase, J. Inherit. Metab. Dis. 16 (1993) 753–761.

[9] E. Chen, W.L. Nyhan, C. Jakobs, C.M. Greco, A.J. Barkovich, V.A. Cox, S. Packman, L-2-Hydroxyglutaric aciduria: neuropathological correlations and first report of severe neurodegenerative disease and neonatal death, J. Inherit. Metab. Dis. 19 (1996) 335–343.

[10] J. Fujitake, Y. Ishikawa, H. Fujii, K. Nishimura, K. Hayakawa, F. Inoue, N. Terada, M. Okochi, Y. Tatsuoka, L-2-hydroxyglutaric aciduria: two Japanese adult cases in one family, J. Neurol. 246 (1999) 378–382.

[11] G.F. Hoffmann, C. Jakobs, B. Holmes, L. Mitchell, G. Becker, H.P. Hartung, W.L. Nyhan, Organic acids in cerebrospinal fluid and plasma of patients with L-2-Hydroxyglutaric aciduria, J. Inherit. Metab. Dis. 18 (1995) 189–193.

[12] H. Watanabe, S. Yamagucgi, K. Saiki et al., Identification of the D-enantiomer of 2-hydroxyglutaric acid in glutaric aciduria type II, Clin. Chim. Acta 238 (1995) 115–124.

[13] R.J.A. Wanders, P. Mooyer, D-2-Hydroxyglutaric acidaemia: identification of a new enzyme, D-2-hydroxyglutarate dehydrogenase, localized in mitochondria, J. Inherit. Metab. Dis. 18 (1995) 194–196.

[14] J.C. Dutra, M. Wajner, C.F. Wannmacher, C.S. Dutra-Filho, C.M.D. Wannmacher, Effects of methylmalonate and propionate on glucose and ketone bodies uptake 'in vitro' by brain of developing rats, Biochem. Med. Metab. Res. 45 (1991) 56–64.

[15] P.A. Trinder, Determination of blood glucose using on oxidase-peroxidase system with a non-carcinogenic chromogen, J. Clin. Pathol. 22 (1969) 158–161.

[16] C.S. Dutra-Filho, M. Wajner, E. Gassen, R. Candiago, A. Wihlelms, H. Malfussi, C.M.D. Wannmacher, Effect of organic acids on in vitro glucose oxidation by cerebral cortex of young rats, Med. Sci. Res. 23 (1995) 25–26.

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

[18] P. Rustin, D. Chretien, T. Bourgeron, B. Gérard, A. Rötig, J.M. Saudubray, A. Munnich, Biochemical and molecular investigations in respiratory chain deficiencies, Clin. Chim. Acta 228 (1994) 35–51.

[19] A.H. Schapira, Nuclear and mitochondrial genetics in Parkinson's disease, J. Med. Genet. 32 (1995) 411–414.

[20] 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.

[21] M. Dixon, E.C. Webb, Enzymes. 2nd Edition, 1964, Longmans, London.

[22] A. Cornish-Bowden, A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors, Biochem. J. 137 (1974) 143–144.

[23] L. D'Incerti, L. Farina, I. Moroni, G. Uziel, M. Savoiardo, L-2-Hydroxyglutaric aciduria: MRI in seven cases, Neuroradiology 40 (1998) 727–733.

[24] R.A. Capaldi, Structure and function of cytochrome C oxidase, Annu. Rev. Biochem. 59 (1995) 418–427.

[25] M.H. Tulinius, E. Holme, B. Kristiansson, N.G. Larsson, A. Oldfors, Mitochondrial encephalomyopathies in childhood, I. Biochemical and morphologic investigations, J. Pediatr. 119 (1991) 242–250.

[26] M. Tulinius, H. Holme, B. Kristiansson, N.G. Larsson, A. Oldfors, Mitochondrial encephalomyopathies in childhood. II. Clinical manifestations and syndromes, J. Pediatr. 119 (1991) 251–259.

[27] M.K. Salo, J. Rapola, H. Somer, H. Pihko, M. Koivileko, H. Tritschler, Oxidase deficiency, Arch. Dis. Child. 67 (1992) 1033–1035.

[28] M. Zerviani, B. Bertagnolia, G. Uziel, Neurological presentation of mitochondrial diseases, J. Inherit. Metab. Dis. 19 (1996) 504–520.

[29] W.T. Lee, P.J. Wang, C. Young, T.R. Wang, Y.Z. Shen, Cytochrome C oxidase deficiency in fibroblasts of a patient with mitochondrial encephalomyopathy, J. Formos. Med. Assoc. 95 (1996) 709–711.

[30] H. Wörle, B. Köhler, W. Schlote, P. Winkler, C.K. Bastanier, Progressive cerebral degeneration of childhood with liver disease (Alpers Huttenlocher disease) with cytochrome oxidase deficiency presenting with epilepsia partialis continua as the first clinical manifestation, Clin. Neuropathol. 17 (1998) 63–68.

[31] J.P. Harpey, D. Heron, M. Prudent, C. Charpentier, P. Rustin, G. Ponsot, V. Cormier Daire, Diffuse leukodystrophy in na infant with cytochrome-c oxidase deficiency, J. Inherit. Metab. Dis. 21 (1998) 748–752.

[32] C. Morin, J. Dub, B.H. Robinson, J. Lacroix, J. Michaud, M. De Braekeleer, G. Geoffroy, A. Lortie, C. Blanchette, M.A. Lambert, G.A. Mitchell, Stroke-like episodes in autosomal recessive cytochrome oxidase deficiency, Ann. Neurol. 45 (1999) 389–392.

[33] T.A. Willis, J. Davidson, R. George, F. Gray, K. Poulton, P. Ramani, W. Whitehouse, Cytochrome oxidase deficiency presenting as birth asphyxia, Dev. Med. Child Neurol. 42 (2000) 414–417.

[34] D.G. Nichols, S.J. Ferguson, Bioenergetics 2, Academic Press, London, 1992, pp. 82–87.

[35] S. Arnold, B. Kadenbach, Cell respiration is controlled by ATP, an allosteric inhibitor of cytochrome *c* oxidase, Eur. J. Biochem. 249 (1997) 350–354.

[36] S. Arnold, B. Kadenbach, Intramitochondrial ATP/ADP ratios control cytochrome *c* oxidase activity allosterically, FEBS Lett. 249 (1999) 105–108.

[37] B. Kadenbach, S. Arnold, A second mechanism of respiratory control, FEBS Lett. 447 (1999) 131–134.

[38] B. Kadenbach, M. Huttelmann, S. Arnold, I. Lee, E. Bender, Mitochondrial energy metabolism is regulated via nuclear-coded subunits of cytochrome *c* oxidase, Free Radic. Biol. Med. 29 (2000) 211–221.

[39] W.S. Kunz, A. Kudin, S. Vielhaber, C.D. Elger, F. Attartdi, G. Villani, Flux control of cytochrome *c* oxidase in human skeletal muscle, J. Biol. Chem. 275 (2000) 27741–27745.

[40] G.F. Hoffman, W. Meier-Augenstein, S. Stocker, R. Surtees, D. Rating, W.L. Nyhan, Physiology and pathophysiology of organic acids in cerebrospinal fluid, J. Inherit. Metab. Dis. 16 (1993) 648–669.

[41] L. Hertz, L. Peng, Energy metabolism at the cellular level of the CNS, Can. J. Physiol. Pharmacol. 70 (1992) 145–157.

[42] R.C. Hennebery, A. Novelli, J.A. Cox, P.G. Lysko, Neurotoxicity at the *N*-methyl-D-aspartate receptor in energy-compromised neurons: a hypothesis for cell death in aging and disease, Ann. N.Y. Acad. Sci. 568 (1989) 225–233.

III. 2. Inibição da citocromo *c* oxidase pelo ácido D-2-hidroxiglutárico em músculo esquelético e músculo cardíaco de ratos jovens

Inhibition of cytochrome *c* oxidase by D-2-hydroxyglutaric acid in skeletal muscle and cardiac muscle of young rats

Cleide G. da Silva, Alethea G. Barschak, Vânia Pulronik, Carlos S. Dutra-Filho, Clóvis M.D. Wannmacher, Ângela T.S. Wyse and Moacir Wajner

Inhibition of cytochrome *c* oxidase activity by D-2-hydroxyglutaric acid in skeletal muscle and cardiac muscle of young rats

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Abstract

Tissue accumulation of high amounts of D-2-hydroxyglutaric acid (DGA) and L-2-hydroxyglutaric acid (LHGA) are the biochemical hallmark of the inherited neurometabolic disorders D-2-hydroxyglutaric aciduria (DHGA) and L-2-hydroxyglutaric aciduria (LHGA) respectively. Although the patients affected by these disorders have predominantly severe neurological findings, aswell as hypotonia, muscular weakness and hypotrophy and miocardiopathy, the underlying mechanisms of muscle injury in these disorders are virtually unknown. In the present study we have evaluated the role of LGA and DGA at concentrations ranging from 0.05-5.0 mM on the activities of the mitochondrial respiratory chain complexes in skeletal and cardiac muscle homogenates of 30-day-old Wistar rats. DGA significantly inhibited cytochrome *c* oxidase activity (complex IV) (EC 1.9.3.1) in a dose-dependent manner (up to 90 %) at doses as low as 0.5 mM, without compromising the other respiratory chain enzyme activities in both muscular tissues. In contrast, LGA did not interfere with these activities. Kinetic studies revealed that the inhibitory effect of DGA was uncompetitive in both tissues. Our results suggest that mitochondrial dysfunction may be an important mechanism involved in the miopathy and miocardiopathy of patients affected by D-2-hydroxyglutaric aciduria.

Keywords: D-2-hydroxyglutaric acid, D-2-hydroxyglutaric aciduria, energy metabolism, respiratory chain, cytochrome c oxidase, skeletal muscle, cardiac muscle.

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1. Introduction

2-Hydroxyglutaric acid is normally excreted in small amounts in the urine of normal individuals and occurs as the D and L enantiomers. However, excessive excretion of D-2-hydroxyglutaric acid (DGA) and L-2-hydroxyglutaric acid (LGA) is characteristically found in D-2-hydroxyglutaric aciduria (DHGA) and L-2-hydroxyglutaric aciduria (LHGA) respectively. DHGA and LHGA are genetic neurometabolic disorders probably transmitted as autossomal recessive traits [1-4]. The underlying biochemical defects of LHGA and DHGA are to date unknown and no specific therapy is available for these conditions.

DHGA was first recognized by Chalmers and coworkers in 1980, and has now been reported in 26 patients [1,2,5,6]. Whereas clinical symptomatology and disease course are homogeneous among the patients with LHGA, the clinical condition in DHGA varies from neonatal-onset intractable epilepsy to mild developmental delay. The most severely affected patients suffered from a rather uniform encephalopathy with developmental delay, epilepsy and hypotonia. Dystonic or choreoathetotic movement disorders and cardiomyopathy with cardiomegaly, hypertrophy and reduced contractility was also observed in many patients affected by this disorder [7-9]. Enlarged frontal subarachnoid spaces and subdural effusions and signs of cerebral delayed maturation with subependymal cysts in the caudate nucleous is generally seen in the MRI. One-third of the patients die during infancy [1,2]. The other variant of DHGA is chacterized by a mild phenotype which essentially shares the characteristics of the severe phenotype [1]. Biochemically, affected patients with DHGA present large urinary excretion and accumulation of D-2-hydroxyglutaric acid (DGA) in plasma and cerebrospinal fluid (CSF) [1,2].

LHGA was first described in 1980 by Duran and colleagues [10] and has now been described in at least 50 patients. Patients affected by this neurodegenerative disorder usually present with psychomotor retardation, mental deterioration, seizures, pyramidal and extrapyramidal symptoms with generally onset in early to mild childhood. Ataxia, intention tremor, choreiform movements and hypotonia are also observed [3,4]. Neuroimaging reveals subcortical leukoencephalopathy, progressive loss of myelinated arcuate fibres, alteration in a basal ganglia and cerebellar atrophy [11-14]. The diagnosis depends upon increased levels of L-2-

hydroxyglutaric acid (LGA) in urine, plasma and cerebrospinal fluid [11].

Besides the high urinary excretion of the enantiomers of 2-hydroglutaric acid, elevated amounts of lactate, 2-ketoglutarate and others Krebs cycle intermediates in urine are also found in some patients with LHGA [12-14] and DHGA [1,2,9]. Therefore, although virtually nothing is known about the pathophysiology of the tissue damage in both pathologies, it is conceivable that a primary or functional mitochondrial dysfunction may be implicated because of the elevated levels of lactate and/or Krebs cycle intermediates observed in the biological fluids of many affected patients.

In this context, tissues that are more dependent on aerobic metabolism, such as the brain, muscle, and heart, are more likely to be affected in these disorders [15] and it is well established that deficiencies in the activities of the enzymes that make up the mitochondrial respiratory chain are associated with a wide range of encephalomyopathic disorders. Indeed, we have recently demonstrated that COX activity is markedly inhibited by DGA in cerebral cortex of rats and human skeletal muscle [16]. Another recent report described that complex V activity of the respiratory chain is blocked by DGA in cultured neurons from chick embryo telencephalos [9].

On the other hand, cardiac involvement has been commonly found in a great number of mitochondrial diseases, either in association with neuromuscular symptoms or, less frequently, as the main clinical feature [17,18]. This is the case for LHGA and particularly for DHGA, in which, besides the predominant central nervous system dysfunction, miopathy with hypotonia, muscle weakness and atrophy, and cardiomiopathy are also important signs [1,2,19,20]. However, to date the factor contributing to muscle pathology in LHGA and DHGA remain unclear. Therefore, we extended our previous study [16] and investigated the in vitro effects of DGA and LGA on respiratory chain enzyme complex activities in heart and skeletal muscle from young rats in the hope to determine whether these acids could compromise energy metabolism in these high energy demand tissues.

2. Materials and Methods

2.1. Subjects and reagents

Thirty-day-old Wistar rats bred our laboratory were used. Rats had free access to water and a 20% (w/w) protein commercial chow and were kept in a room with a 12:12 –h light/dark cycle and temperature of 24 ± 1°C. The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA. D-2-hydroxyglutaric acid (DGA) and L-2-hydroxyglutaric acid (LGA) were dissolved in the specific buffer used for each enzyme technique.

2.2. Homogenate preparation

Animals were killed by decapitation without anesthesia, the skeletal muscle and cardiac muscle were dissected onto an ice-cold glass plate and homogenized using an ice-chilled glass homogenizing vessel at 900 rpm (10 strokes). Rat skeletal muscle and cardiac muscle were homogenized (1:20, w/v) in SETH buffer, pH 7,4 (250 mM sucrose, 2mM EDTA, 10 mM Trizma base, 50 UI.mL^{-1} heparin). The homogenates were centrifuged at 800 x g for 10 minutes and the supernatants kept at -70°C until used for the enzyme activity determination. The maximal period between homogenate preparation and enzyme analysis was always less than 5 days.

2.3. Respiratory chain enzyme activities

The activities of respiratory chain enzyme complexes succinate-DCIP-oxioredutase (complex II) (EC 1.3.5.1), succinate:cytochrome c oxidoreductase (complex II + CoQ + complex III) (EC 1.10.2.2) and the activity of succinate: phenazine oxireductase (soluble succinate dehydrogenase-SDH) were determined in homogenates according to the method of Fischer et al. [21].

Complex II (succinate-DCIP-oxireductase) activity was measured by following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1$ mM^{-1}. cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8.0 µM DCIP was preincubated with 40-80 µg homogenate protein at 30ºC for 20 min. Subsequently, 4.0 mM sodium azide and 7.0 µM rotenone were added and the reaction was initiated by addition of 40 µM DCIP and was monitored for 5 minutes at 30°C.

The activity of succinate: phenazine oxireductase (soluble succinate dehydrogenase- SDH) was measured following the decrease in absorbance due to the reduction of DCIP at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1$ mM^{-1}. cm^{-1}) in the presence of phenazine methasulphate (PMS). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8.0 µM DCIP was preincubated with 40-80 µg homogenate protein at 30ºC for 20 min. Subsequently, 4 mM sodium azide, 7.0 µM rotenone and 40 µM DCIP were added. After 5 minutes the reaction was initiated by addition of 1.0 mM PMS and was monitored for 5 minutes at 30°C.

Complex II + III activity was measured by following the increase in absorbance due to the reduction of cytochrome c at 550 nm with 580 nm as the reference wavelength ($\varepsilon = 19.1$ mM^{-1}. cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate was preincubated with 40-80 µg homogenate protein at 30ºC for 30 min. Subsequently, 4.0 mM sodium azide and 7.0 µM rotenone were added and the reaction was initiated by the addition of 0.6 µg/mL cytochrome c and monitored for 5 minutes at 30°C.

Skeletal Muscle
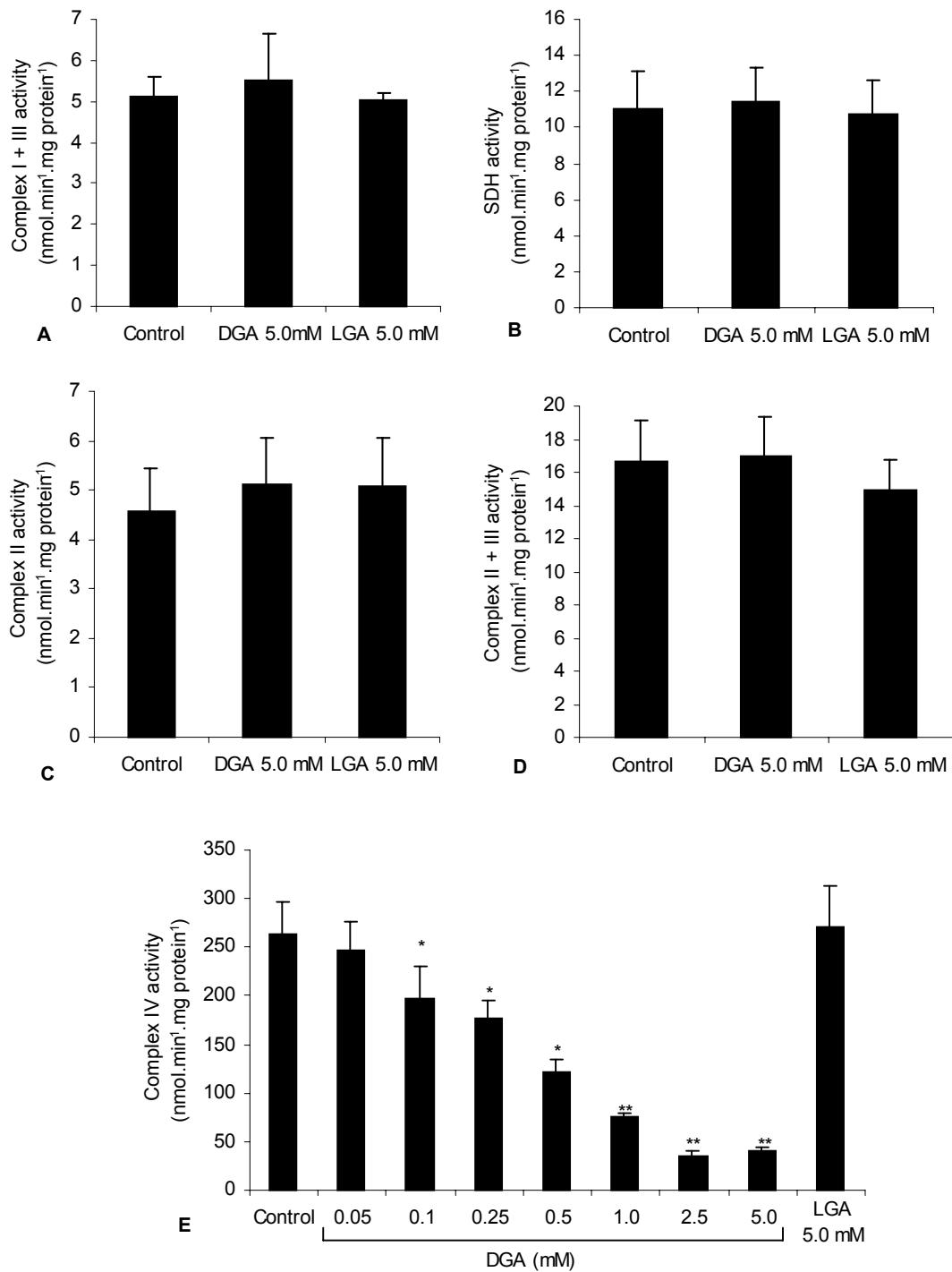
Figure 1: Effect of D-2-hydroxyglutaric acid (DGA) and L-2-hydroxyglutaric acid (LGA) on the activities (nmol.min^{-1}.mg protein^{-1}) of respiratory chain in skeletal muscle homogenates of 30-day-old rats. Values are mean ± S.E.M. for n=5 independent experiments per group of the respiratory chain complexes activities measured in the presence or absence of the metabolites. * p< 0.05, ** p< 0.01 compared to controls (Duncan multiple range test).

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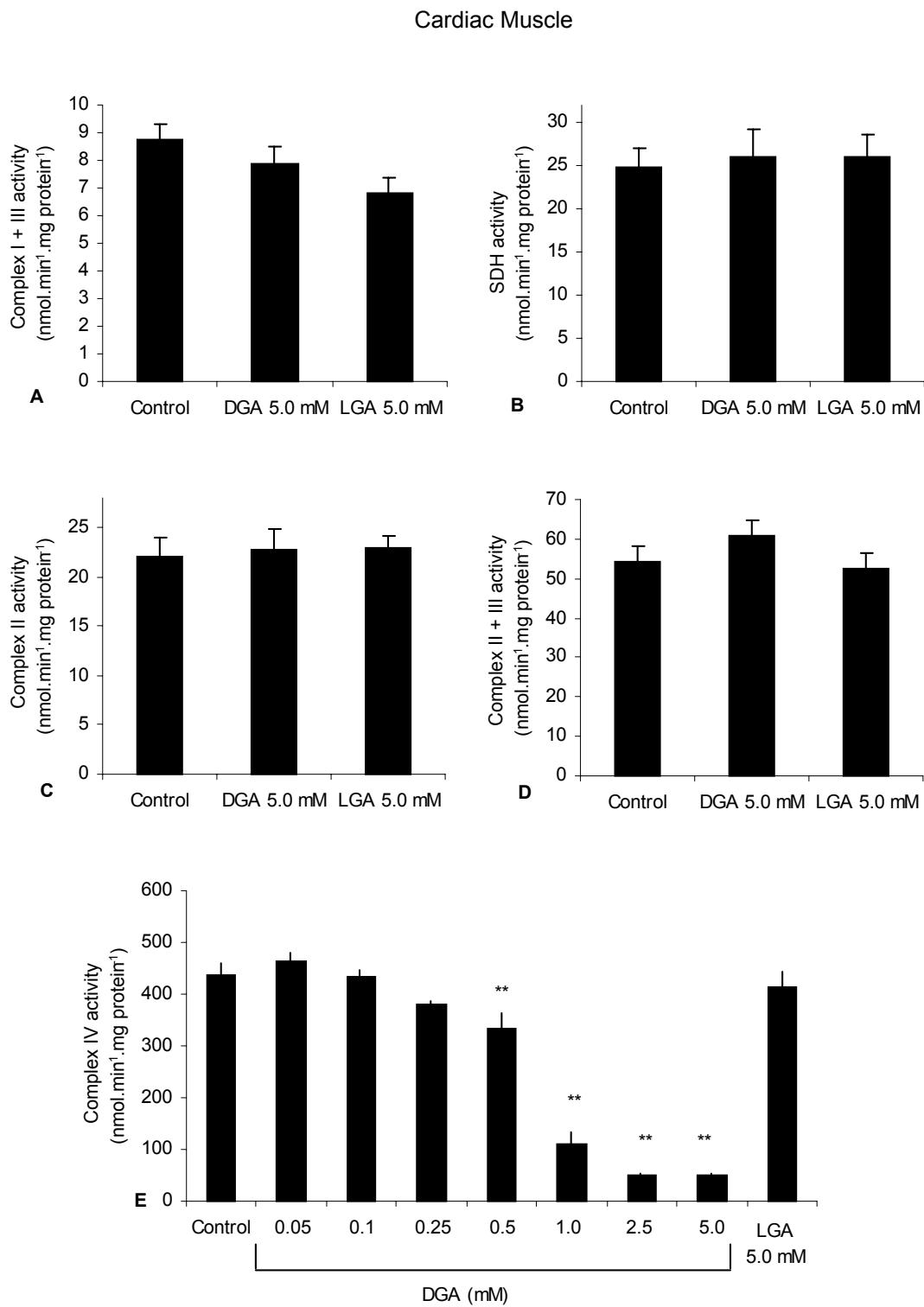
Cardiac Muscle

Figure 2: Effect of D-2-hydroxyglutaric acid (DGA) and L-2-hydroxyglutaric acid (LGA) on the activities (nmol.min^{-1}.mg protein^{-1}) of respiratory chain in cardiac muscle homogenates of 30-day-old rats. Values are mean ± S.E.M. for n=4-8 independent experiments per group of the respiratory chain complexes activities measured in the presence or absence of the metabolites. ** $p < 0.01$ compared to controls (Duncan multiple range test).
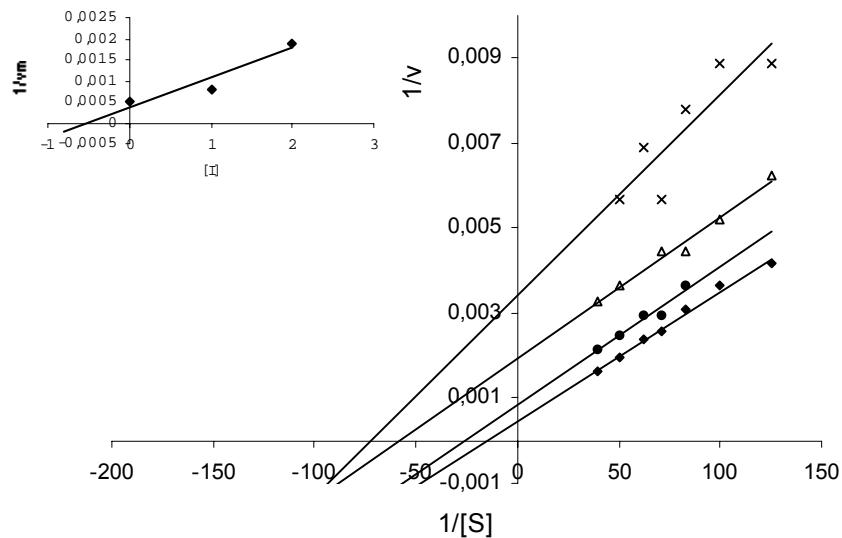
Skeletal Muscle

Figure 3: Kinetic analysis of the inhibition of cytochorme *c* oxidase (COX) from skeletal muscle of rats by D-2-hydroxyglutaric acid (DGA). The graph shows a double plot of COX activity (nmol.min^{-1}.mg protein[-1]) for cytochorme *c* concentrations (0.008 – 0.025 μM) ([S]) in the absence (♦, controls) and in the presence of 1.0 (●), 2.0 (Δ) and 2.5 (x) mM DGA ([I]). The insert shows the Dixon plot for Ki determination. All experiments were repeated at least three times and similar results were obtained. Data presented are representative of one experiment.

The activity of cytochrome *c* oxidase (complex IV) (COX) (EC 1.9.3.1) was measured according to Rusting et al. [22], whereas that of NADH:cytochrome *c* oxidoreductase (complex I + CoQ + complex III) were assayed according to the method described by Shapira et al. [23].

Complex IV activity was measured by following the decrease in absorbance due the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength (ε=19.1 mM[-1].cm^{-1}). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM n-dodecyl-β-D-maltoside, 2-4 μg homogenate protein and the reaction was initiated with addition of 0.7 μg reduced cytochrome *c*. The activity of complex IV was measured at 25ºC for 10 minutes.

Complex I + CoQ + III activity was measured by following the increase in absorbance due to reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength (ε=19.1 mM[-1].cm^{-1}). The reaction buffer contained 20 mM potassium phosphate, pH 8.0, 2 mM KCN, 10 μM EDTA, 50 μM cytochrome *c* and 10-25 μg homogenate protein. The reaction was initiated by addition of 25 μM NADH and was monitored at 30ºC for 3 min before addition of 10 μM rotenone, after witch the activity was measured for an additional 3 min. Complex I + CoQ + III activity was the rotenone-sensitive NADH: cytochrome *c* reductase activity.

Respiratory chain activities were determined in the presence of various concentrations of DGA or LGA (0.5

to 5.0 mM). Control groups did not contain any acid in the incubation medium.

2.4. Protein determination

Protein was measured by the method of Lowry et al. [24] using serum albumin bovine as standard.

2.5. Statistical analysis

The results are presented as means ± standard error of the mean. Data from the activities of the respiratory chain enzyme complexes were analyzed by one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when *F* was significant. Linear regression analysis was also used to test dose-dependent effects. Differences between groups were rated significant at a probability error less than 0.05.

3. Results

We first determined the effect of DGA and LGA on the activities of the respiratory chain enzyme complexes in homogenates of rat skeletal muscle (figure 1). DGA strongly reduced the activity of COX (up to 86.9%) [$F_{(8,44)}$=14.823, p<0.0001)] in a dose-dependent way [β=-0.880, p<0.0001]. In contrast, LGA did not alter this activity. The activities of complex I+III [$F_{(2,14)}$=0.114, p>0,05], complex II [$F_{(2,14)}$=0.101, p>0,05], succinate dehydrogenase (SDH) [$F_{(2,14)}$=0.027, p>0,05] and

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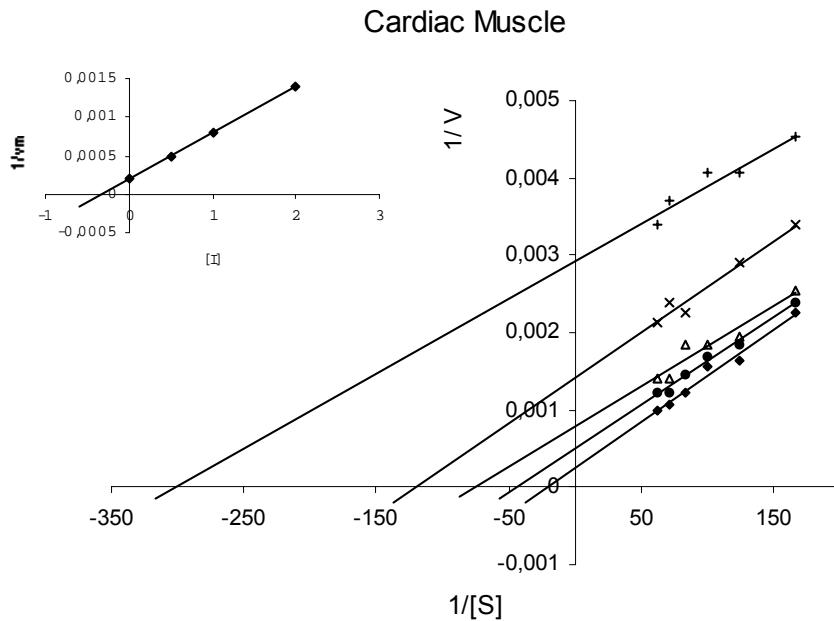
Figure 4: Kinetic analysis of the inhibition of cytochorme *c* oxidase (COX) from skeletal muscle of rats by D-2-hydroxyglutaric acid (DGA). The graph shows a double plot of COX activity (nmol.min^{-1}.mg protein^{-1}) for cytochrome *c* concentrations (0.006 – 0.016 μM) ([S]) in the absence (♦, controls) and in the presence of 0.5 (●), 1.0 (Δ), 2.0 (x) and 3.0 (+) mM DGA ([I]). The insert shows the Dixon plot for *K*i determination. All experiments were repeated at least three times and similar results were obtained. Data presented are representative of one experiment.

complex II+III [F(2,14)=0.242, p>0,05] were not affected by DGA and LGA.

We then investigated the effect of DGA and LGA on respiratory chain enzyme complexes activities from homogenates of rat cardiac muscle (figure 2). It can be seen that DGA significantly inhibited (up to 89,2%) COX activity [F(8,35)=61,599, p<0.0001] in a dose dependent manner [β=-0.905, p<0.0001], whereas DGA did not affect complex I+III [F(2,23)=3.104, p>0,05], complex II [F(2,14)=0.080, p>0,05], succinate dehydrogenase (SDH) [F(2,14)=0.072, p>0,05] and complex II+III [F(2,14)=1.115, p>0,05] activities. Furthermore, LGA did not alter any of the enzymatic complex activities tested in cardiac muscle. The concentration required to inhibit 50% of COX activity in both tissues examined corresponded to the IC$_{50}$ [25]. The data indicated an IC$_{50}$= 0.312 ± 0.075mM and 0.794 ± 0.26 for skeletal muscle and cardiac muscle, respectively.

The kinetics of the interaction of DGA with COX in homogenates from skeletal muscle and cardiac muscle was also determined [26]. The Lineweaver-Burk double reciprocal plot was analyzed over a range of cytochrome *c* concentrations (0.006 – 0.025 μM) in the absence or presence of DGA (0.5 – 3.0 mM). The data indicate that the inhibition of COX activity by DGA is uncompetitive in both tissues (figure 3 and 4). The Km calculated was 0.059 ± 0.016 mM and 0.05 ± 0.001mM (mean ± S.D., n=3) for skeletal muscle and cardiac muscle, respectively. The *K*i value (the dissociation constant of the enzyme-substrate-inhibitor complex) was calculated by the method of Dixon [25], which provides a simple way of

determining the inhibitor constant (*K*i) for uncompetitive inhibitors. The *K*i calculated was 0.56 ± 0,017 mM for skeletal muscle and 0.465 ± 0.177 mM for cardiac muscle (mean ± S.D., n=3).

4. Discussion

Besides the significant neurological findings, patients with DHGA and LHGA commonly present muscular symptoms, such as hypotonia and weakness, which may be associated with muscular atrophy. In addition, cardiomyopathy is frequently associated with the severe form of DHGA [1-4,7]. However, very little known about the pathogenic mechanisms underlying the tissue damage in these diseases. Although tissue accumulation of DGA and LGA are the biochemical hallmark of DHGA and LHGA, respectively, in some of the documented patients the elevation of these metabolites is accompanied by increased excretion of lactate and citric acid cycle intermediates in urine and this is indicative of energy metabolism impairment.

In this contest, we recently demonstrated that COX activity is markedly inhibited by DGA in rat cerebral cortex and human skeletal muscle [16]. We extended this study and showed in the present report that DGA strongly inhibited COX activity in a concentration-dependent manner in skeletal and cardiac muscle of young rats, whereas the other activities of the respiratory chain were not changed by the metabolite. The kinetic analysis revealed that this inhibition was uncompetitive. In contrast, LGA did not alter any of the respiratory chain

complex activities studied, indicating that DGA effect was specific rather than a nonspecific effect of acidic compounds. Furthermore, as regards LGA, our results are in agreement with a previous report demonstrating that the activities of complexes I to IV of the respiratory chain are normal in liver and muscle biopsies from patients with LHGA, indicating that the sustained tissue elevation of LGA does not provoke inhibition of these activities [12].

Generation of energy in eukaryotic cells is largely dependent on oxidative phosphorilation (OXPHOS), where the major part of cellular ATP is produced. The human cytochrome c oxidase complex (COX) is one of five mitochondrial multisubunit complexes responsible for the process of oxidative phosphorilation. COX is a multicomponent membrane protein complex of molecular size 200 kD made up of 13 protein components, 10 of which are encoded in the nucleus and the core the cytochrome complex is made up from three protein encoded in the mitochondrial DNA [27]. COX function is to take electrons from cytochrome c generated from substrate oxidation and deliver it to molecular oxygen which is reduced to water. In the process, protons are pumped from the mitochondrial matrix into the intermembrane space, contributing to generate the electrochemical proton gradient across the inner mitochondrial membrane which is used to drive ATP synthesis via the F_1F_0-ATPase (complex V) [28,29].

OXPHOS activity is usually controlled by the amount of substrates and the electrochemical proton gradient across the membrane [30]. However, it has been recently demonstrated that the rate of the respiratory chain and ATP synthesis can be also effectively regulated by a mechanism based on the allosteric inhibition of COX at high intramitochondrial ATP/ADP ratios [29,31-33]. Other studies demonstrated that COX regulation is based on reversible and cAMP dependent phosphorylation of the nuclear-code subunits of COX via Ca^{2+} -activated protein phosphatases [34] and hypothesized that this may represent a general mechanism of energy metabolism control based on variable efficiency of energy transduction in COX and on the turning on and off of respiratory control via the intramitochondrial ATP/ADP ratio [29]. Therefore, it has been hypothesized that disruption of this important enzyme complex of the mitochondrial respiratory chain, which corresponds to the last site of ATP synthesis would seriously affect energy production in the cell [35].

On the other hand, human COX deficiency is frequently associated with lactic acidemia and may occur as distinct phenotypic forms [28]. In general, the main clinical features of COX deficiency include hypotonia, lethargy, feeding difficulties, failure to thrive, psychomotor delay, seizures, and progressive deterioration of basal ganglia and brain stem [36,37]. The most severe type is a fatal infantile COX deficiency that causes death from lactic acidosis in the newborn period and presents with a renal Fanconi syndrome and myopathy [38,39]. Another phenotype is myopathy with hypertrophic cardiomyopathy. The neonatal miopathic presentation of this form of COX deficiency is characterized by generalized hypotonia, weakness, respiratory insufficiency, and lactic acidosis [15]. Fibroblasts from affected patients presents raised lactate to pyruvate ratio and COX activity of 35 to 45% [36,40-42]. These laboratorial and clinical signs are similar to those found in DHGA, a fact that possibly indicates that the marked inhibition (90 %) of COX activity observed in the present study may potentially be implicated in the pathophysiology of the neuromuscular findings presented by patients affected by DHGA. This is further reinforced by the kinetic analysis of DGA-induced COX inhibition which showed that the concentration of DGA necessary to decrease 50 % (IC_{50}) of COX activity and that the Ki values in skeletal muscle and cardiac muscle were of the micromolar order. Therefore, it is expected that this degree of inhibition would probably disrupt energy metabolism in these tissues.

Furthermore, because myocardium depends heavily on oxidative metabolism, the COX inhibition by DGA in this work might have an important role in the cardiopathy present in the severe form of DHGA. However, further studies are needed to test the in vivo effect of DGA on COX activity.

In conclusion, it is conceivable that a severe reduction of COX activity in skeletal and cardiac muscle caused by DGA as found in the present study may impair energy production in these tissues and be related to the myopathy and cardiomyopathy observed in patients affected by DHGA. It may also explain the elevated levels of lactate and citric acid intermediates in the body fluid of some of these patients, which may occur secondarily to the inhibition of the respiratory chain electron flux leading to an increase of NADH and FADH2 concentrations. Elevation of these reduced nucleotides would possibly increase lactate by the high $NADH/NAD^+$ ratio and inhibit succinate and alpha-ketoacid dehydrogenase leading therefore to the accumulation of their precursors succinate and alpha-glutaric acid.

Acknowledgements

References

[1] van der Knaap MS, Jakobs C, Hoffman GF et al. D-2-Hydroxyglutaric aciduria: further clinical delineation. J Inherit Metab Dis 1999; 22:404-413.

[2] van der Knaap MS, Jakobs C, Hoffmann GF et al. D-2-Hydroxyglutaric aciduria: biochemical marker or clinical disease entity? Ann Neurol 1999; 45:111-119.

[3] Barth PG, Hoffman GF, Jaeken J et al. L-2-hydroxyglutaric acidaemia: a novel inherited neurometabolic disorder. Ann Neurol 1992; 32:66-71.

[4] Barth PG, Hoffmann GF, Jaeken J et al. L-2-hydroxyglutaric academia: Clinical and biochemical findings in 12 patients and preliminary report on L-2-hydroxyacid dehydrogenase. J Inher Metab Dis 1993; 16:753-761.

[5] Chalmers RA, Lawson AM, Watts RWE et al. D-2-Hydroxyglutaric aciduria: case report and biochemical studies. J Inherit Metab Dis 1980; 3:11-15.

[6] Wajner M, Vargas CR, Funayama C et al. D-2-hydroxyglutaric aciduria in a patient with a severe clinical phenotype and unusual MRI findings. J Inherit Metab Dis 2002; 25:28-34.

[7] Eeg-Olofsson O. D-2-hydroxyglutaric aciduria with cerebral, vascular, and muscular abnormalities in a 14-year-old boy. J Child Neurol 2000; 15:488-492.

[8] Sugita K, Kakinuma H, Okajima Y et al. Clinical and MRI findings in a case report of D-2-hydroxyglutaric aciduria. Brain Dev 1995; 17:139-141.

[9] Kölker S, Pawlak V, Ahlemeyer B et al. NMDA receptor activation and respiratory chain complex V inhibition contribute to neurodegeneration in D-2-hydroxyglutaric aciduria. Eur J Neurosci 2002; 16:21-28.

[10] Duran M, Kamerling JP, Barkker HD, van Gennip HA, Wadman SK, L-2-hydroxyglutaric aciduria: an inborn error of metabolism? J Inherit Metab Dis 1980; 3:11-15.

[11] Barbot C, Fineza I, Diogo L et al. L-2-hydroxyglutaric aciduria: clinical, biochemical and magnetic resonance imaging in six Portuguese pediatric patients. Brain Dev 1997; 19:268-273.

[12] Barth PG, Wanders RJ, Scholte HR et al. L-2-Hydroxyglutaric aciduria and lactic acidosis. J Inherit Metab Dis 1998; 21:251–254

[13] Chen E, Nyahn WL, Jakobs C et al. L-2-hydroxyglutaric aciduria: neuropathological correlations and first report of severe neurodegenerative disease and neonatal death. J Inherit Metab 1996; 19:335-343.

[14] Hoffmann GF, Jakobs C, Holmes B et al. Organic acids in cerebrospinal fluid and plasma of patients with L-2-Hydroxyglutaric aciduria. J Inherit Metab Dis 1995; 18:189–193

[15] Sue CM, Hirano M, DiMauro S, De Vivo DC. Neonatal presentations of mitochondrial metabolic disorders. Semin Perinatol 1999; 23:113-124.

[16] Silva CG, Ribeiro CAJ, Leipnitz G et al. Inhibition of cytochrome *c* oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid in vitro. Biochim Biophys Acta 2002; 1586:81-91.

[17] Bohlega S, Tanji K, Santorelli F et al. Multiple mitochondrial DNA deletions associated with autosomal recessive ophthalmoplegia and severe cardiomyopathy. Neurology 1996; 46:1329-1334.

[18] Anan R, Nakagawa M, Miyata M. et al.. Cardiac involvement in mitochondrial diseases. Circulation 1995; 91:955-961.

[19] Nyhan WL, Shelton GD, Jacobs C et al. D-2-Hydroxyglutaric aciduria. J Child Neurol 1995; 10:137-142.

[20] Baker NS, Sarnat HB, Jack RM et al. D-2-hydroxyglutaric aciduria: hypotonia, cortical blindness, seizures, cardiomyophathy and cylindrical spirals in skeletal muscle. J Child Neurol 1997; 12:31-36

[21] Fischer JC, Ruitenbeek W, Berden JA et al. Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin Chim Acta 1985; 153: 23-36.

[22] Rustin P, Chretien D, Bourgeron T et al. Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 1994; 228: 35-51.

[23] Schapira AHV, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 1990; 54: 823-827.

[24] Lowry OH, Rosebrough NJ, Farr AL, Randall R J. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-267.

[25] Dixon, M. and Webb, E.C. Enzymes. 2nd Edition, 1964. Longmans, London.

[26] Cornish-Bowden, A. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem J 1974; 137:143-144.

[27] Capaldi RA. Structure and function of cytochrome oxidase. Ann Rev Biochem 1992; 59:569-596.

[28] Rodinson BH. Human cytochrome oxidase deficiency. Pediatr Res 2000; 48:581-585.

[29] Kadenbach B, Hüttemann M, Arnold S, Lee I and Bender E. Mitochondrial energy metabolism is regulated via nuclear-coded subunits of cytochrome *c* oxidase. Free Radic Biol Med 2000; 29:211-221.

[30] Nichols DG, Ferguson SJ. Bioenergetics 2, London: Academic Press, 1992.

[31] Arnold S, Kadenbach B. Cell respiration is controlled by ATP, an allosteric inhibitor of cytochrome c oxidase. Eur J Biochem 1997; 249:350-354.

[32] Arnold S, Kadenbach B. Intramitochondrial ATP/ADP ratios control cytochrome c oxidase activity allosterically. FEBS Lett 1999; 443:105-108.

[33] Kadenbach B, Arnold S. A second mechanism of respiratory control [minireview]. FEBS Lett 1999; 447:131-134.

[34] Bender E, Kadenbach B. The allosteric ATP inhibition of cytochrome c oxidase is reversible switched on the camp-dependent phosphorilation. FEBES Lett 2000; 466:730-734.

[35] Fry M, Green D. Cardiolipin requirement by cytochrome *c* oxidase and the catalytic role of phospholin. Biochem Biophys Res Commun 1980; 93:1238–1248.

[36] Van Coster R, Lombes A, DeVivo DC et al. Cytochrome c oxidase-associated Leigh syndrome: phenotypic features and pathogenic speculations. J Neurol Sic 1991; 104:97-111.

[37] Lombes A, Nakase H, Tritschler HJ et al. Biochemical and molecular analysis of cytochrome c oxidase deficiency in Leigh's syndrome. Neurology 1991; 41:491-498.

[38] DiMauro S, Mendell JR, Sahenk Z et al. Fatal infantile mitochondrial myophaty and renal dysfunction due to cytochrome c oxidase deficiency. Neurology 1980; 30:795-804.

[39] Minchom PE, Dormer RL, Hughes IA et al. Fatal infantile mitochondrial myopathy due cytochrome c oxidase deficiency. J Neurol Sci 1983; 60:453-463.

[40] Robinson BH. Lacticacidemia: biochemical, clinical, and genetic considerations. In: Harris H, Hirschhorn K, editors. Advances in human genetics. Plenum Press, New York, 1989: 151-179.

[41] Glerum DM, Robinson BH, Capaldi RA. Fibroblats and cytohrome c oxidase deficiency. In: Azzi A, Drahota Z, Papa S, editors. Molecular basis of membrane associated diseases. Springer-Verlag, Berlin, 1989: 228-238.

[42] Shoubridge EA. Cytochorme c oxidase deficiency. Am J Med Genet 2001; 106: 46-52.

III. 3. Inibição da atividade da creatina quinase em córtex cerebral de ratos pelo ácido D-2-hidroxiglutárico *in vitro*

Inhibition of creatine kinase activity from rat cerebral cortex by D-2-hydroxyglutaric acid *in vitro*

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Inhibition of creatine kinase activity from rat cerebral cortex by D-2-hydroxyglutaric acid in vitro

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Abstract

D-2-Hydroxyglutaric acid (DGA) is the biochemical hallmark of patients affected by the neurometabolic disorder known as D-2-hydroxy-glutaric aciduria (DHGA). Although this disease is predominantly characterized by severe neurological findings, the underlying mechanisms of brain injury are virtually unknown. In the present study, we investigated the effect of DGA on total, cytosolic, and mitochondrial creatine kinase (CK) activities from cerebral cortex of 30-day-old Wistar rats. Total CK activity (tCK) was measured in whole cell homogenates, whereas cytosolic and mitochondrial activities were measured in the cytosolic and mitochondrial preparations from cerebral cortex. We verified that CK activities were significantly inhibited by DGA (11–34% inhibition) at concentrations as low as 0.25 mM, being the mitochondrial fraction the most affected activity. Kinetic studies revealed that the inhibitory effect of DGA was non-competitive in relation to phosphocreatine. We also observed that this inhibition was fully prevented by pre-incubation of the homogenates with reduced glutathione, suggesting that the inhibitory effect of DGA on tCK activity is possibly mediated by oxidation of essential thiol groups of the enzyme. Considering the importance of CK activity for brain metabolism homeostasis, our results suggest that inhibition of this enzyme by increased levels of DGA may be related to the neurodegeneration of patients affected by DHGA.
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Keywords: D-2-Hydroxyglutaric acid; Brain metabolism; Creatine kinase

1. Introduction

D-2-Hydroxyglutaric aciduria (DHGA) is a rare neurometabolic disorder biochemically characterized by tissue accumulation and high urinary excretion of D-2-hydroxyglutaric acid (DGA; Chalmers et al., 1980; Van der Knaap et al., 1999a,b). It was first recognized by Chalmers et al. (1980) and has now been reported in at least 25 patients (Van der Knaap et al., 1999b). It is clinically characterized by at least two variants, a severe form with early-infantile-onset encephalopathy, whose patients usually present with seizures, hypotonia, and poor development, as well as enlarged frontal subarachnoid spaces and subdural effusions and signs of cerebral delayed maturation with subependymal cysts in the caudate nucleus detected by MRI. The other variant is clinically milder and more variable, presenting usually with mental retardation, macrocephaly and hypotonia. Delayed cerebral maturation, ventriculomegaly, and subependymal cysts are usually observed by MRI. Biochemically, besides the high excretion of DGA, lactate and Krebs cycle intermediates are also found in elevated amounts in the urine of a considerable number of patients (Van der Knaap et al., 1999a,b). High excretion of DGA also occurs in multiple acyl-CoA dehydrogenase deficiency (glutaric acidemia type II), which is due to a defect of the electron transfer flavoprotein (ETP) or of the mitochondrial enzyme ETF-ubiquinone oxidoreductase (Watanabe et al., 1995).

Although the metabolic pathways of DGA is probably of importance to the central nervous system, the underlying biochemical defect of DHGA and the origin of this

metabolite have remained an enigma. Moreover, virtually nothing is known about the pathophysiology of the neurological dysfunction of DHGA, and the effects of DGA on cell metabolism are practically unknown and await investigation. However, considering that elevated levels of lactate and/or Krebs cycle intermediates are observed in some patients affected by this disorder, it is conceivable that a mitochondrial dysfunction may be associated with this disease.

Creatine kinase (CK) is found in high concentrations only in neuromuscular tissues, which are characterized by an up to 10-fold increase of ATP turnover within milliseconds during excitation (Shainberg et al., 1971). During these rapid changes, creatine/phoshocreatine (Cr/PCr) is necessary as an energy buffering and transferring system to avoid large fluctuations of cellular ATP/ADP levels (Bessman and Carpenter, 1985; Wallimann et al., 1992). CK catalyses the reversible transphosphorylation between ATP and creatine to ADP and phosphocreatine (CK reaction: $MgATP + \text{creatine} \leftrightarrow \text{phosphocreatine} + MgADP + H^+$), which helps to balance the levels of the phosphorylated substrates.

CK consists of a group of five isoenzymes, being three cytosolic dimers (Cy-CK), BB-CK (brain-specific), MM-CK (muscle-specific), and the heterodimer CK-MB (present only in cardiac muscle cytosol). There are also two additional mitochondrial forms of the enzyme (Mi-CK), one positioned in the intermembrane space, termed Mib-CK (specific for sarcomeric muscle) and the isoform Mia-CK (the ubiquitous form), which is present in the brain (Wallimann et al., 1992).

Due to its location near the sites where energy generation and ion transport across membranes take place, the CK/phosphocreatine system seems to play a complex multi-faceted role in neuromuscular energy homeostasis. Therefore, it is not surprising that impaired CK function leads to the development of various pathological states involving the brain, skeletal muscle and cardiac muscle (Hamman et al., 1995; Liao et al., 1996; David et al., 1998; Aksenov et al., 1999, 2000).

In a previous study, we have demonstrated that DGA compromises brain energy production by decreasing glucose uptake, CO_2 production and strongly inhibiting cytochrome *c* oxidase activity in cerebral cortex of young rats without affecting the other activities of the respiratory chain (Silva et al., 2002).

Therefore, considering the close interrelation between oxidative phosphorylation and the CK/phosphocreatine system, the present study was designed to test the in vitro effects of DGA on total CK activity (tCK), as well as on CK activities in cytosolic (Cy-CK) and mitochondrial (Mi-CK) preparations from cerebral cortex of young rats in order to clarify the pathogenetic mechanisms of brain injury of DHGA. We also tested the influence of the antioxidant reduced glutathione (GSH), the free radical scavengers ascorbic acid and α-tocopherol and the nitric oxide synthase (NOS) inhibitor *N*ω-nitro-L-arginine methyl ester (L-NAME) on the

effects produced by DGA on tCK activity since the enzyme contain thiol groups which can be oxidized and are critical to its function.

2. Experimental procedures

2.1. Subjects and reagents

Wistar rats obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúda, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, were housed in groups of eight with their mothers on the day of birth. They were maintained on a 12 h:12 h light/dark cycle (lights on 07:00–19:00 h) in an air-conditioned constant temperature ($22 \pm 1\,°C$) colony room, with free access to water and a 20% (w/w) protein commercial chow (Germani, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, and followed the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985).

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). DGA was dissolved in Tris–HCl buffer, pH 7.5, for the enzymatic technique.

2.2. Preparation of total homogenates and the mitochondrial and cytosolic fractions

For the preparation of total homogenates, 30-day-old Wistar rats were sacrificed by decapitation without anesthesia, the brain was rapidly removed, pons, medulla, and cerebellum were discarded, and the cerebral cortex was dissected and homogenized in 10 volumes of saline solution, pH 7.5, using a ground glass type Potter-Elvejhem homogenizer immersed in an ice box. At least two homogenizations of 30-s duration were performed at approximately 1000 ms/min with an electrically driven Teflon pestle. This homogenate was used for the estimation of tCK activity. Mitochondrial and cytosolic fractions were prepared according to Ramirez and Jiménez (2000). The homogenates were centrifuged at $800 \times g$ for 10 min at $4\,°C$ and the pellet discarded. The supernatant was then centrifuged at $27,000 \times g$ for 30 min at $4\,°C$ in a Sorval DC-2B centrifuge. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the enzymatic assay. The supernatants were further centrifuged at $125,000 \times g$ for 60 min at $4\,°C$ in an OTD-65B Sorval centrifuge, the microsomal pellet discarded, and the cytosol (supernatant) was used for the enzymatic assay. The cytosolic fraction did not contain contaminating Mi-CK, as observed by immunoblotting. The samples were stored at $-40\,°C$ and the maximal period between total homogenate and subcellular preparations and enzyme analysis was always less than 5 days. We have previously

observed that under these conditions CK activity is not altered.

2.3. Creatine kinase activities

CK activity was measured in total homogenates, as well as in the cytosolic and mitochondrial preparations from cerebral cortex. The reaction mixture consisted of the following medium: 60 mM Tris–HCl buffer, pH 7.5, containing 7 mM phosphocreatine, 9 mM $MgSO_4$, and approximately 0.4–1.2 μg protein in a final volume of 0.1 ml. For enzymatic analysis in mitochondrial fractions, 0.625 mM lauril maltoside was added to the incubation mixture. DGA at 0.25–5 mM final concentrations was also supplemented to the medium, whereas controls did not contain the acid. After 15 min of pre-incubation at 37 °C, the reaction was started by the addition of 3.2 mM ADP plus 0.8 mM reduced GSH. The reaction was stopped after 10 min by the addition of 1 μmol *p*-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls containing all components of the incubation medium except homogenates, that is CK, were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 ml 2% α-naphthol and 0.1 ml 0.05% diacetyl in a final volume of 1 ml and read after 20 min at 540 nm. In some experiments, the homogenates were pre-incubated with 0.5 mM GSH, 1 mM L-NAME, 1 mM ascorbic acid, or 10 μM α-tocopherol (trolox) final concentrations for 15 min alone or in the presence of the metabolite in 60 mM Tris–HCl buffer, pH 7.5, containing 7 mM phosphocreatine and 9 mM $MgSO_4$, after which the other reagents were supplemented and reaction carried out. None of the substances supplemented to the assay medium interfered with the color development. Results were expressed as μmol creatine formed/min mg protein.

2.4. Protein determination

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

2.5. Statistical analysis

Unless otherwise stated, results are presented as means ± S.D. All assays were performed in duplicate and the mean was used for the calculations. Data from the activity of CK were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc Duncan multiple range test when F was significant. For analysis of dose-dependent effect, linear regression was used. The Student's *t* test was also used for comparison of two means. Differences between the 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.
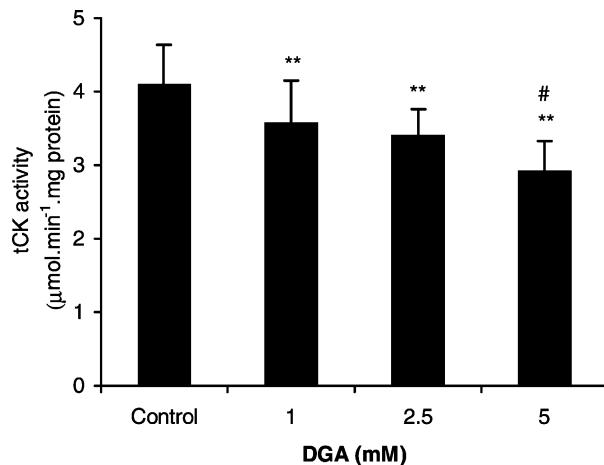
Fig. 1. Effect of DGA on tCK activity (μmol creatine/min mg protein) in cerebral cortex homogenates of 30-day-old rats. Values are means ± S.D. for $n = 9$ independent experiments per group of CK activity measured in cerebral cortex homogenates in the presence or absence of the metabolite. One-way ANOVA ($F_{(3, 35)} = 8.861$; $P < 0.0001$) followed by the Duncan's multiple range test (** $P < 0.01$ compared to control; # $P < 0.05$ compared to 2.5 and 1.0 mM DGA).

3. Results

We first examined the effect of DGA, at concentrations varying from 1 to 5 mM, on tCK activity in whole homogenates from cerebral cortex. Fig. 1 shows the results of such experiments. Values for these activities in controls were similar to those described previously (O'Gorman et al., 1996). It can be seen that DGA significantly inhibited tCK activity (13–28%) from cerebral cortex at all concentrations tested and in a dose-dependent manner ($\beta = -0.663$, $P < 0.0001$) (Fig. 1).

We then investigated the effect of DGA at concentrations varying from 0.25 to 5 mM on CK activity from the mitochondrial (Mi-CK) and cytosolic (Cy-CK) fractions of rat cerebral cortex (Figs. 2 and 3). It can be seen that the metabolite significantly inhibited Mi-CK activity (14–34%) in a dose-dependent fashion ($\beta = -0.613$, $P < 0.0001$) (Fig. 2), and Cy-CK activity (11–21%) in a similar fashion ($\beta = -0.869$, $P < 0.0001$) (Fig. 3).

The next step was to study the kinetics of the interaction of DGA with CK in total homogenates from cerebral cortex (Cornish-Bowden, 1974). The Lineweaver–Burk double-reciprocal plot was analyzed over a range of phosphocreatine concentrations (0.5–2 mM) in the absence or presence of DGA (1–5 mM). The data indicate that the inhibition of CK activity by DGA is non-competitive (Fig. 4). The K_m calculated for tCK was 2.02 ± 0.3 mM ($n = 3$). The K_i value (the dissociation constant of the enzyme–substrate–inhibitor complex) was calculated by the method of Dixon (Dixon and Webb, 1964), which provides a simple way of determining the inhibition constant (K_i)
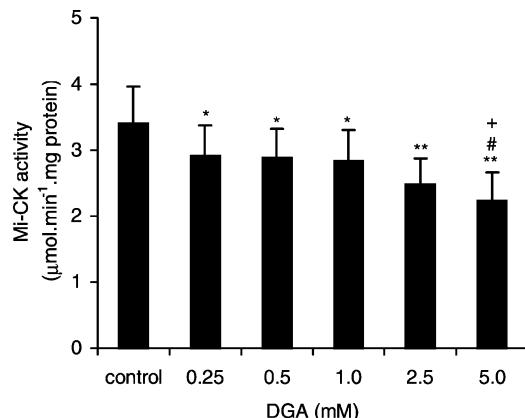
Fig. 2. Effect of DGA on CK activity (μmol creatine/min mg protein) in mitochondrial preparations (Mi-CK) from cerebral cortex of 30-day-old rats. Values are means ± S.D. for $n = 9$ independent experiments per group of Mi-CK activity measured in the presence or absence of the metabolite. One-way ANOVA ($F(5, 43) = 6.729$; $P < 0.0001$) followed by the Duncan's multiple range test ($^*P < 0.05$, $^{**}P < 0.01$ compared to control; $^\#P < 0.05$ compared to 1.0, 0.5, and 0.25 mM DGA; $^+P < 0.01$ compared to 0.5 and 0.25 mM DGA).

Fig. 3. Effect of DGA on CK activity (μmol creatine/min mg protein) in cytosolic preparations (Cy-CK) from cerebral cortex of 30-day-old rats. Values are means ± S.D. for $n = 6$ independent experiments per group of Cy-CK activity measured in the presence or absence of the metabolite. One-way ANOVA ($F(5, 35) = 6.805$, $P < 0.0001$) followed by the Duncan's multiple range test ($^*P < 0.05$ compared to control; $^\#P < 0.05$ compared to 0.5 and 0.25 mM DGA).

243 for non-competitive inhibitors. The K_i value calculated for
244 DGA in tCK was 10.1 ± 1.06 mM ($n = 5$).
245 In order to test whether the significant reduction of CK
246 activity caused by DGA was due to a non-specific effect
247 of D-enantiomers, we then investigated the effect of 5 mM
248 α-ketoglutaric acid (KGA), another dicarboxylic acid with

249 similar chemical structure to DGA, on tCK in homogenates.
250 KGA did not affect CK activity in homogenates from
251 cerebral cortex (control: 1.750 ± 0.249 μmol/min mg pro-
252 tein; KGA: 1.763 ± 0.193 μmol/min mg protein ($n = 6$);
253 $t(10) = 0.14$, $P = 0.919$). Furthermore, the effects of
3-hydroxyglutaric and glutaric acids (0.1–5 mM), com-
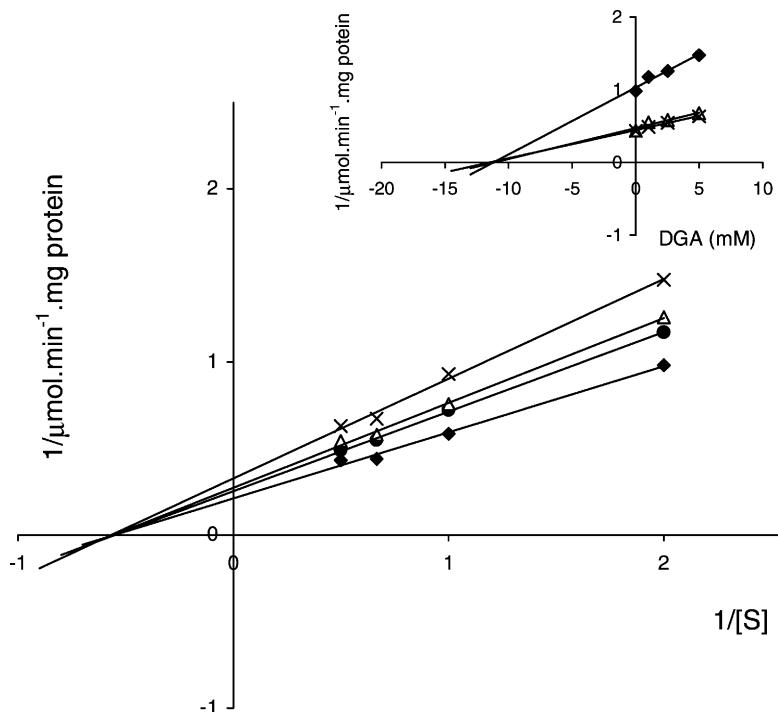
Fig. 4. Kinetic analysis of the inhibition of tCK from cerebral cortex of rats by DGA. The graph shows a double-reciprocal plot of CK for phosphocreatine concentrations (0.5–2.0 mM) [S] in the absence (◆, controls) and in the presence of 1.0 (●), 2.5 (△), and 5.0 (×) mM DGA. The inset shows the Dixon plot for K_i determination. All experiments were repeated at least three times and similar results were obtained. Data presented are representative of one experiment.

Table 1
Total CK activity in cerebral cortex homogenates exposed to 3-hydroxylglutaric acid and glutaric acid

Control	3-Hydroxyglutaric acid (mM)		
	0.1	1.0	–
3.15 ± 0.29	2.87 ± 0.41	2.88 ± 0.32	–
	Glutaric acid (mM)		
	0.1	1.0	5.0
3.56 ± 0.33	3.43 ± 0.47	3.96 ± 0.79	3.80 ± 0.68

Data represent the mean $\pm$ S.D. for $n = 6$ independent experiments performed in triplicate and are expressed as μmol/min mg protein. Comparison between means of the various groups revealed no significant differences (one-way ANOVA: 3-hydroxyglutaric acid: $F(2, 17) = 1.23$; $P > 0.05$; glutaric acid: $F(3, 20) = 0.925$; $P > 0.05$).

pounds with a molecular mass similar to that of DGA, on tCK activity were also evaluated in order to test whether the significant reduction of CK activity caused by DGA was due to a non-specific effect of acidic compounds. We observed that both acids caused no alteration on this activity in cerebral cortex homogenates (Table 1). We finally tested the effect of DGA (1.0 mM) on Na^+,K^+-ATPase from rat cerebral cortex and observed that the acid did not alter this enzyme activity (results not shown). Taken together, our data suggest that the inhibitory effect of DGA on this enzyme activity is probably specific.

We further evaluated whether the DGA inhibitory activity was mediated by oxidation of critical (–SH) groups on the enzyme, by pre-incubating cerebral cortex homogenates in the presence of the antioxidant GSH, the free radical scavengers, ascorbic acid and α-tocopherol, and the NOS inhibitor L-NAME. The enzyme activity was measured in the whole cell homogenates since free radicals can be better produced with the whole cell machinery. The concentrations of the antioxidants used in these assays were similar to those previously described (Stanimirovic et al., 1995; Avrova et al., 1998; Kölker et al., 2002b). First, by pre-incubating cerebral cortex homogenates in the presence of ascorbic acid (1 mM) or the NOS inhibitor L-NAME (1 mM), alone or combined with 5 mM DGA, we observed that ascorbate and L-NAME per se had no effect on tCK activity, in contrast to DGA which significantly inhibited the enzyme activity. Furthermore, ascorbic acid and L-NAME per se did not alter tCK activity neither prevented the inhibitory effect of DGA on this activity (Fig. 5). We also tested the effects of the naturally occurring antioxidant agent GSH (0.5 mM) and the free radical scavenger α-tocopherol (10 μM) on tCK activity, and verified that these drugs per se did not affect tCK activity, but GSH fully prevented the inhibitory effect of DGA towards this enzyme activity (Fig. 6). These results indicate that reduction of sulfhydryl groups on the enzyme by antioxidants protect tCK activity, and that nitric oxide or other common free radicals were not involved in the inhibition.
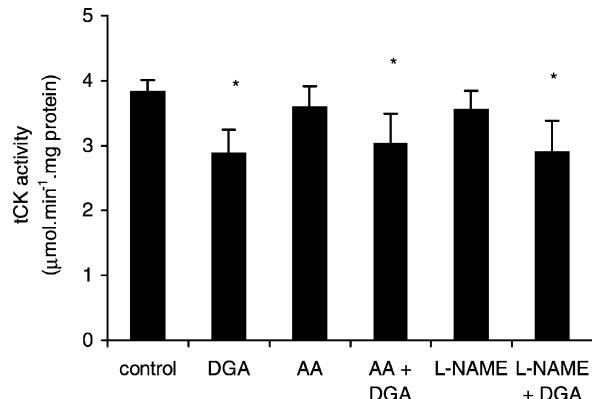
Fig. 5. Effect of ascorbic acid (AA) and L-NAME on cerebral cortex tCK activity in the presence or absence of DGA. Cortical homogenate preparations were pre-incubated at 37 °C for 15 min with 5.0 mM DGA, 1 mM AA, 5 mM DGA plus 1 mM AA, 1 mM L-NAME, or 5.0 mM DGA plus 1 mM L-NAME, and the enzyme activity was determined afterwards. Data are means $\pm$ S.D. for $n = 6$ independent experiments. One-way ANOVA ($F(5, 30) = 7.365$, $P < 0.0001$) followed by the Duncan's multiple range test ($^*P < 0.05$ compared to control).
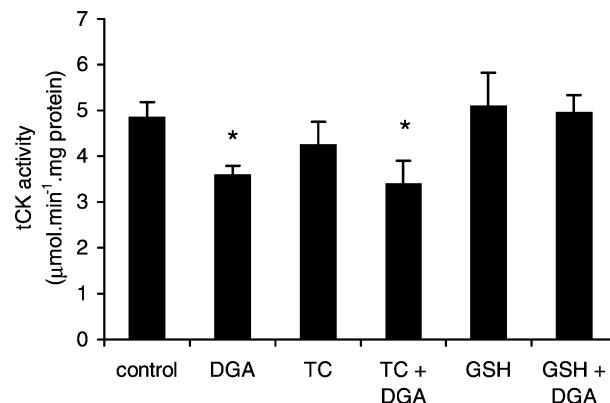
Fig. 6. Effect of α-tocopherol (TC) and reduced GSH on cerebral cortex tCK activity in the presence or absence of DGA. Cortical homogenates were pre-incubated at 37 °C for 15 min with 5.0 mM DGA, 10 μM TC, 5.0 mM DGA plus 10 μM TC, 0.5 mM GSH, or 5.0 mM DGA plus 0.5 mM GSH, and the enzyme activity was determined afterwards. Data are means $\pm$ S.D. for $n = 5$ independent experiments. One-way ANOVA ($F(5, 29) = 11.71$, $P < 0.001$) followed by the Duncan's multiple range test ($^*P < 0.05$ compared to control).

4. Discussion

Tissue accumulation and urinary excretion of high concentrations of DGA is the biochemical characteristic of DHGA. Although the symptomatology of this disorder is predominantly neurological and marked white-matter atrophy of the brain is frequently found in the affected patients, very little is known about the pathogenetic mechanisms underlying the brain damage of DHGA.

Recent studies from our and other laboratories indicate a compromised brain energy production and/or utilization caused by DGA (Silva et al., 2002; Kölker et al., 2002a).

We have shown that DGA, at concentrations of 0.5 mM and higher, strongly inhibits glucose uptake, CO_2 production, and cytochrome *c* oxidase activity without compromising the other respiratory chain enzyme activities in rat cerebral cortex and human skeletal muscle homogenates (Silva et al., 2002). Kölker et al. (2002a) found that DGA significantly inhibits complex V activity of the respiratory chain in cultured neurones from chick embryo telencephalons and neonatal rat hippocampus. These investigators also showed that DGA induces ROS generation, which was significantly attenuated by the NMDA inhibitor MK-801, suggesting that this effect was at least partially mediated via activation of the NMDA receptors, rather than being a direct effect of DGA.

On the other hand, neurological symptoms are common in patients affected by disorders presenting reduction of CK activity (David et al., 1998; Aksenov et al., 1999, 2000) and impaired cardiac energetics and skeletal muscle alterations of the expression of critical proteins involved in energy homeostasis are found in CK knockout mice models (Saupe et al., 1998; Groof et al., 2001).

Therefore, considering that CK, especially Mi-CK, is functionality coupled to the mitochondrial adenine nucleotide translocator (ANT) and oxidative phosphorylation (OXPHOS), in the present study, we investigated the influence of various concentrations of DGA on the activities of rat brain CK, a key enzyme necessary for normal brain energy metabolism.

We initially observed that DGA significantly inhibited tCK activity in cerebral cortex homogenates by up to 30% in a dose-dependent manner. We also demonstrated that DGA significantly reduced Mi-CK activity (by up to 34%) and Cy-CK activity (by up to 21%) in the cerebral cortex of the animals. The kinetic analysis of the effects of DGA on tCK activity indicated a non-competitive inhibition. The K_i values obtained for the inhibition of CK by DGA with respect to phosphocreatine in total homogenates were of the millimolar order and within the intracellular phosphocreatine concentrations in the brain (Bergmeyer, 1974).

Furthermore, our observations that KGA, glutaric, and 3-hydroxyglutaric acids, compounds of similar molecular structure and mass to that of DGA, caused no alteration of CK activity point to a specific effect of DGA, rather than a non-specific action due to acidic or dicarboxylic substances. These results also indicate that in our system conditions DGA is not mainly converted to KGA since, if that was the case, no alteration of CK activity would be detected. Furthermore, DGA also did not affect Na^+,K^+-ATPase activity from rat cerebral cortex, reinforcing the selective inhibitory action of DGA towards CK.

Our results indicate inhibitory actions of DGA towards brain Mi-CK activity, which is mainly the Mia-CK isoform, and to the brain-specific Cy-CK activity (BB-CK). Investigation of the effect of DGA on the mitochondrial (Mib-CK) and cytosolic (MM-CK and MB-CK) isoenzymes from skeletal and cardiac muscle was not carried out in the present work, but this may be of significance since affected patients also have myopathy (Eeg-Olofsson, 2000) and cardiomyopathy (Van der Knaap et al., 1999a,b).

The molecular defect of DHGA is still unknown. However, some of the documented DHGA patients accumulate and excrete increased amounts of lactate and citric acid cycle intermediates or dicarboxylic acids, a fact that indicates a primary or functional mitochondrial dysfunction (Van der Knaap et al., 1999b). In this context, it has been postulated that DHGA could be due to a primary mitochondrial defect, possibly a separate ETF-linked or ETF-ubiquinone oxireductase-linked dehydrogenase (Van der Knaap et al., 1999a). However, the previous studies from our and other laboratory showing a blockage of the respiratory chain at complexes IV and V caused by DGA (Silva et al., 2002; Kölker et al., 2002a,b) and the present data demonstrating a significant inhibition of CK activity, more pronounced for the mitochondrial isoform, point to a secondary mitochondrial dysfunction caused by the metabolite which most accumulates in this disorder. The elevation in the levels of Krebs cycle intermediates found in some of the patients affected by DHGA may, therefore, occur secondarily to the blockage of the respiratory chain electron flux leading to an increase of NADH and $FADH_2$ concentrations. Increase of these reduced nucleotides may possibly lead to inhibition of KGA and succinate dehydrogenases and consequently leading to an increase of their precursors α-ketoglutarate and succinate which appear increased in their concentration in this disease. Lactate may also be increased because of the high $NADH/NAD^+$ ratio. In this context, it would be important to determine whether the DGA levels are more elevated in patients excreting or accumulating these intermediates.

We also investigated the influence of various antioxidants on the inhibitory effect of DGA on brain tCK activity since this molecule has cysteine residues in its structure that can be a target for NO and other free radicals which inactivate the enzyme (Yuan et al., 1992; Mekhfi et al., 1996; Wolosker et al., 1996; Stachowiak et al., 1998; Konorev et al., 1998). We verified that GSH that acts as a naturally occurring thiol-reducing agent (Meister and Anderson, 1983) fully prevented the inhibitory role of DGA on total brain CK activity. Therefore, it is likely that this inhibition may be mediated by oxidation of sulfhydryl or other groups of the enzyme. These results are in agreement with other studies using Mi-CK from cardiac muscle and showing that the free-radical induced inhibition of this enzyme activity can be reversed in vitro by the reducing agents dithiothreitol and 2-mercaptoethanol (Gross et al., 1996), indicating the involvement of the active-site essential cysteine 278 residue of CK (Yuan et al., 1992). On the other hand, α-tocopherol, which is an excellent trapping agent for lipid peroxyl radicals ($ROO^\bullet$) (Burton et al., 1990), ascorbic acid, which traps hydroxyl and superoxide radicals (Halliwell and Gutteridge, 1999), and the NOS inhibitor L-NAME did not prevent DGA inhibitory activity towards brain tCK. These findings, therefore, are indicative that DGA possibly acts through oxida-

tion of critical thiol or other groups present on the enzyme and necessary to its function. Furthermore, it can be also concluded that, under our in vitro assay conditions, NO or other common free radicals are not involved in the inhibitory effect of DGA on brain CK isoforms, since we used high doses of the NOS inhibitor, L-NAME, and of the free radical scavengers, ascorbic acid and α-tocopherol. Our present results are also of interest because, to our knowledge, to date nothing has been described on the influence of the redox potential on the brain CK activity or on the influence of antioxidants on active-site groups or other groups of the enzyme that can be oxidized.

The CK system is crucial for brain cells being recognized as an important metabolic regulator during health and disease (Wallimann et al., 1998). In this context, decreased brain CK activity of the same degree as that observed in the present study is found in various neurodegenerative diseases, including Alzheimer's disease (Tomimoto et al., 1993; Hensley et al., 1995; Aksenov et al., 1997, 2000) and creatine and phosphocreatine supplementation have neuroprotective effects against energy deprivation and glutamate excitotoxicity probably due to an increase of cytosolic high-energy phosphate stores (Holtzman et al., 1999; Matthews et al., 1999; Malcon et al., 2000; Shear et al., 2000).

It should be also emphasized that significant inhibition of CK activities was achieved with 0.25 mM and higher doses of DGA, which are within the blood circulating and CSF (46–757 μmol/l) levels of DGA in patients affected by DHGA (Van der Knaap et al., 1999a,b). Although the brain concentrations of DGA in these patients are yet unknown, we cannot exclude that even higher intracerebral concentrations of DGA may be attained in this neurometabolic disease, as possibly occur in other similar organic acidemias so-called "cerebral" organic acidemias whose symptoms are predominantly or almost exclusively neurological (Hoffman et al., 1993). Therefore, if the results presented in this report can be extrapolated to the human condition, it is conceivable that these concentrations may affect energy utilization and/or production in the brain of these patients. However, additional studies are necessary to test the in vivo effect of DGA on CK activities, and this investigation is currently being undertaken in our laboratory.

Whether impairment of brain energy utilization or other biochemical abnormalities is the principal underlying mechanism responsible for the encephalopathy of DHGA patients remains to be elucidated. Finally, we propose that products that improve the energy status of the cells such as creatine and maintain the redox state of neural cells such as GSH esters and pro-cystein drugs, which can penetrate into the brain, should be carefully considered as potential adjuvant therapy for these patients.

References

Aksenov, M.I., Aksenova, M.V., Payne, R.M., Smith, C.D., Markesbery, W.R., Carney, J.M., 1997. The expression of creatine kinase isoenzymes in neocortex of patients with neurodegenerative disorders: Alzheimer's and Pick's disease. Exp. Neurol. 146, 458–465.

Aksenov, M., Aksenova, M.V., Payne, R.M., Trojanovski, J.Q., Schmidt, K.L., Carney, J.M., Butterfield, D.A., Markesbery, W.R., 1999. Oxidation of cytosolic proteins and expression of creatine kinase BB in frontal lobes of Neurodegenerative disorders. Dement. Geriatr. Cogn. Disord. 10, 158–165.

Aksenov, M., Aksenova, M.V., Butterfield, A.D., Markesbery, W.R., 2000. Oxidative modification of creatine kinase BB in Alzheimer's disease brain. J. Neurochem. 74, 2520–2527.

Avrova, N.F., Victorov, I.V., Tyurin, V.A., Zakharova, I.O., Sokolova, T.V., Andreeva, N.A., Stelmaschuk, E.V., Tyurina, Y.Y., Gonchar, V.S., 1998. Inhibition of glutamate-induced intensification of free radical reactions by gangliosides: possible role in their protective effect in rat cerebellar granule cells and brain synaptosomes. Neurochem. Res. 23, 945–952.

Bergmeyer, H.U., 1974. Methods of Enzymatic Analysis, 2nd ed. Academic Press, New York and London.

Bessman, S.P., Carpenter, C.L., 1985. The creatine–creatine phosphate energy shuttle. Annu. Rev. Biochem. 54, 831–862.

Burton, G.W., Wronska, U., Stone, L., Foster, D.O., Ingold, K.U., 1990. Biokinetics of dietary RRR-α-tocopherol in the male guinea-pig at three dietary levels of vitamin C and two levels of vitamin E. Lipids 25, 199–210.

Chalmers, R.A., Lawson, A.M., Watts, R.W.E., Tavill, A.S., Kamerling, J.P., Hey, E., Ogilvie, D., 1980. D-2-Hydroxyglutaric aciduria: case report and biochemical studies. J. Inherit. Metab. Dis. 3, 11–15.

Cornish-Bowden, A., 1974. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem. J. 137, 143–144.

David, S.S., Shoemaker, M., Haley, B.E., 1998. Abnormal properties of creatine kinase in Alzheimer's disease brain: correlation of reduced enzyme activity and active site photolabelling with aberrant cytosol-membrane partitioning. Mol. Brain Res. 54, 276–287.

Dixon, M., Webb, E.C., 1964. Enzymes, 2nd ed. Longmans, London.

Eeg-Olofsson, O., 2000. D-2-Hydroxyglutaric aciduria with cerebral, vascular and muscular abnormalities in a 14-year-old boy. J. Child Neurol. 15, 488–492.

Groof, A.J.C., Smeets, B., Groot Koerkamp, M.J.A., Mul, A.N., Janssen, E.E.W., Tabak, H.F., Wieringa, B., 2001. Changes in mRNA expression profile underlie phenotypic adaptations in creatine kinase-deficient muscles. FEBS Lett. 506, 73–78.

Gross, W.L., Bak, M.I., Ingwall, J.S., Arstall, M.A., Smith, T.W., Balligand, J.L., Kelly, R., 1996. Nitric oxide inhibits creatine kinase and regulates heart contractile reserve. Proc. Natl. Acad. Sci. U.S.A. 93, 5604–5609.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine, 3rd ed. Oxford University Press, London.

Hamman, B.L., Bittl, J.A., Jacobus, W.E., Aleen, P.D., Spencer, R.S., Tina, R., Ingwall, J.S., 1995. Inhibition of creatine kinase reaction decrease the contractile reserve of isolated rat hearts. Am. J. Physiol. 269, H1030–H1036.

Hensley, K., Hall, N., Subramaniam, R., Cole, P., Harris, M., Aksenova, M.V., Aksenov, M.Y., Gabbita, S.P., Carney, J.M., Lowell, M., Markesbery, W.R., Betterfield, D.A., 1995. Brain regional correspondence between Alzheimer's disease histopathology biomarkers of protein oxidation. J. Neurochem. 65, 2146–2156.

Hertz, L., Peng, L., 1992. Energy metabolism at the cellular level of the CNS. Can. J. Physiol. Pharmacol. 70, 145–157.

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

Holtzman, D., Khait, I., Mulken, R., Albred, E., Rand, T., Jensenm, F., Kraft, R., 1999. In vivo development of brain phosphocreatine in normal and creatine-treated rabbits pups. J. Neurochem. 73, 2477–2484.

Hughes, B.P., 1962. A method for estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin. Chim. Acta 7, 597–604.

Kölker, S., Pawlak, V., Ahlemeyer, B., Okun, J.G., Horster, F., Mayatepek, E., Krieglstein, J., Hoffmann, G.F., Kohr, G., 2002a. NMDA receptor activation and respiratory chain complex V inhibition contribute to neurodegeneration in D-2-hydroxyglutaric aciduria. Eur. J. Neurosci. 16, 21–28.

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

Konorev, E., Hogg, N., Kalyanaraman, B., 1998. Rapid and irreversible inhibition of creatine kinase by peroxynitrite. FEBS Lett. 427, 171–174.

Liao, R., Nascimben, L., Friedrich, J., Gawthmey, J.K., Ingwall, J.S., 1996. Decrease energy reserve in an animal model of dilated cardiomyopathy. Relationship to contractile performance. Circ. Res. 78, 893–902.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–267.

Malcon, C., Kaddurah-Daoh, R., Beal, M.F., 2000. Neuroprotective effects of creatine administration against NMDA and malonate toxicity. Brain Res. 860, 195–198.

Matthews, R.T., Ferrante, R.J., Klivenji, P., Yang, L., Klein, A.M., Mueller, G., Kaddwrah-Daouk, R., Beal, M.F., 1999. Creatine and cyclocreatine attenuate MPTP neurotoxicity. Exp. Neurol. 157, 142–149.

Meister, A., Anderson, M.E., 1983. Glutathione. Annu. Rev. Biochem. 52, 711–760.

Mekhfi, H., Veksler, V., Mateo, P.H., Maupoil, V., Rochette, L., Ventura-Clapier, R., 1996. Creatine kinase in the main target of reactive oxygen species in cardiac myofibrils. Circ. Res. 17, 1016–1027.

O'Gorman, E., Beutner, G., Wallimann, T., Brdiczka, D., 1996. Differential effects of creatine depletion on the regulation of enzyme activities and on creatine-stimulated mitochondrial respiration in skeletal muscle. Biochim. Biophys. Acta 1276, 161–170.

Ramirez, O., Jiménez, E., 2000. Opposite transitions of chick brain catalytically active cytosolic creatine kinase isoenzymes during development. Int. J. Dev. Neurosci. 18, 815–823.

Saks, V.A., Kuznetsov, A.V., Kuprianov, V.V., Miceli, M.V., Jacobus, W.E., 1985. Creatine kinase of rat heart mitochondria. The demonstration of functional coupling to oxidative phosphorylation in an inner membrane-matrix preparation. J. Biol. Chem. 260, 7757–7764.

Saupe, K.W., Spindler, M., Tian, R., Ingwall, J.S., 1998. Impaired cardiac energetics in mice lacking muscle-specific isoenzymes of creatine kinase. Circ. Res. 82, 898–907.

Shainberg, A., Yagil, G., Yaffe, D., 1971. Alterations of enzymatic activities during muscle differentiation in vitro. Dev. Biol. 25, 1–29.

Shear, D.A., Haik, K.L., Dunbar, G.L., 2000. Creatine reduces 3-nitropropionic-acid-induced cognitive and motor abnormalities in rats. Neuroreport 11, 1833–1837.

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

Stachowiak, O., Dolder, M., Wallimann, T., Richter, C., 1998. Mitochondrial creatine kinase is prime target of peroxynitrite-induced modification and inactivation. J. Biol. Chem. 273, 16694–16699.

Stanimirovic, D.B., Wong, J., Ball, R., Durkin, J.P., 1995. Free radical-induced endothelial membrane dysfunction at the site of blood–brain barrier: relationship between lipid peroxidation, Na,K-ATPase activity, and 51Cr release. Neurochem. Res. 20, 1417–1427.

Tomimoto, H., Yamamoto, K., Homburger, H.A., Yanagihara, T., 1993. Immunoelectron microscopic investigation of creatine kinase BB-isoenzyme after cerebral ischemia in gerbils. Acta Neuropathol. 86, 447–455.

Van der Knaap, M.S., Jakobs, C., Hoffmann, G.F., Nyhan, W.L., Renier, W.O., Smeitink, J.A.M., Catsman-Berrevoets, C.E., Hjalmarson, O., Vallance, H., Sugita, K., Bowe, C.M., Herrin, J.T., Craigen, W.J., Buist, N.R.M., Brookfield, D.S.K., Chalmers, R.A., 1999a. D-2-Hydroxyglutaric aciduria: biochemical marker or clinical disease entity? Ann. Neurol. 45, 111–119.

Van der Knaap, M.S., Jakobs, C., Hoffman, G.F., Duran, M., Muntau, A.C., Schweitzer, S., Kelley, R.I., Parrot-Rouland, F., Amiel, J., De Lonlay, P., Rabier, D., Eeg-Olofsson, O., 1999b. D-2-Hydroxyglutaric aciduria: further clinical delineation. J. Inherit. Metab. Dis. 22, 404–413.

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., 1992. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phospho-creatine circuit' for cellular energy homeostasis. Biochem. J. 28, 121–140.

Wallimann, T., Doler, M., Schlattner, U., Eder, M., Hornemann, T., Kraft, T., Stolz, M., 1998. Creatine kinase: an enzyme with central role in cellular energy metabolism. Magn. Reson. Mater. Phys. Biol. Med. 6, 116–119.

Watanabe, H., Yamagucgi, S., Saiki, K., Shimizu, N., Fukao, T., Kondo, N., Orii, T., 1995. Identification of the D-enantiomer of 2-hydroxyglutaric acid in glutaric aciduria type II. Clin. Chim. Acta 238, 115–124.

Wolosker, H., Panizzutti, R., Englender, S., 1996. Inhibition of creatine kinase with *S*-nitrosoglutathione. FEBS Lett. 392, 274–276.

Yuan, G., Kaneko, M., Masuda, H., Hon, R.G., Kobayashi, A., Yamazak, N., 1992. Decrease in heart mitochondrial creatine kinase activity due to oxygen free radical. Biochim. Biophys. Acta 140, 78–84.

III. 4. Inibição da atividade da creatina quinase mitocondrial pelo ácido D-2-hidroxiglutárico em cerebelo de ratos jovens

Inhibition of mitochondrial creatine kinase activity by D-2-hydroxyglutaric acid in cerebellum of young rats

Cleide G. da Silva, Ana Rúbia F. Bueno, Patrícia F. Schuck, Guilhian Leipnitz, César A.J. Ribeiro, Rafael B. Rosa, Carlos S. Dutra-Filho, Ângela T.S. Wyse, Clóvis M.D. Wannmacher and Moacir Wajner

Inhibition of Mitochondrial Creatine Kinase Activity by D-2-Hydroxyglutaric Acid in Cerebellum of Young Rats

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D-2-hydroxyglutaric aciduria (DHGA) is a neurometabolic disorder biochemically characterized by tissue accumulation and excretion of high amounts of D-2-hydroxyglutaric acid (DGA). Although the affected patients have predominantly severe neurological findings, the underlying mechanisms of brain injury are virtually unknown. In previous studies we have demonstrated that DGA, at concentrations as low as 0.25 mM, significantly young rats. In the present study, we investigated the effect of DGA (0.25 – 5 mM) on total creatine kinase (tCK) activity, as well as on CK activity in cytosolic (Cy-CK) and mitochondrial (Mi-CK) preparations from cerebellum of 30-day-old Wistar rats in order to test whether the inhibitory effect of DGA on CK was tissue specific. We verified that tCK (22% inhibition) and Mi-CK (40% inhibition) activities were moderately inhibited by DGA at concentrations of 2.5 mM and higher, in contrast to Cy-CK, which was not affected by the acid. Decreased creatine kinase activity and other parameters of energy metabolism in cerebral cortex of Kinetic studies revealed that the inhibitory effect of DGA was non-competitive in relation to phosphocreatine. We also observed that this inhibition was fully prevented by preincubation of the homogenates with reduced glutathione, suggesting that the inhibition of CK activity by DGA is possibly mediated by modification of essential thiol groups of the enzyme. Our present results therefore demonstrate a relatively weak inhibitory effect of DGA on cerebellum Mi-CK activity, as compared to that provoked in cerebral cortex, and may possibly be related to the neuropathology of DHGA, characterised by cerebral cortex abnormalities.

KEY WORDS: D-2-hydroxyglutaric acid, D-2-hydroxyglutaric aciduria, brain metabolism, mitochondrial creatine kinase.

INTRODUCTION

D-2-hydroxyglutaric aciduria (DHGA) is a rare neurometabolic disorder biochemically characterized by tissue accumulation and high urinary excretion of D-2-hydroxyglutaric acid (DGA) (1-3). DHGA was first recognized by Chalmers and coworkers in 1980, and has now been reported in 26 patients (2-5). It is clinically characterized by at least two variants, a severe form with early-infantile-onset encephalopathy, whose patients usually present with seizures, hypotonia and poor development, as well as delayed cerebral maturation, ventriculomegaly and subependymal cysts observed by magnetic resonance imaging (MRI). The other variant is clinically milder and more variable, presenting usually with mental retardation, macrocephaly and hypotonia. Biochemically, besides the high excretion of DGA, lactate and Krebs cycle intermediates are also found in elevated amounts in the urine of a considerable number of patients (2,3).

Despite the intensive clinical investigation, the underlying biochemical defect of this disorder and the origin of DGA have remained an enigma. Moreover, virtually nothing is known about the pathophysiology of the neurological dysfunction of DHGA. However, considering that elevated levels of lactate and/or Krebs cycle intermediates are observed in some patients (2,3), it is conceivable that a primary or functional mitochondrial dysfunction may be associated with this disease. In this context, we have previously demonstrated that DGA compromises brain energy production by decreasing glucose uptake, CO_2 production, as well as cytochrome *c* oxidase activity without affecting the activity of the other complexes of the respiratory chain in cerebral cortex of young rats (6).

Creatine kinase (CK, EC 2.7.2.3) is a key enzyme of cellular metabolism. It is involved in ATP homeostasis in

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cells with high and fluctuating energy demand, catalyzing the reversible phosphorylation of creatine with ATP. Creatine kinases comprise a group of five oligomeric isoforms with tissue-specific expression and isoenzyme-specific subcellular localization (7). The mitochondrial isoforms of CK are the muscle sarcomeric Mi_b-CK and the ubiquitous Mi_a-CK, which is mainly found in brain mitochondria (8). Mi-CK resides in the mitochondrial intermembrane space, is believed to occur as an octamer in vivo (9) and has preferential access to ATP exported from the mitochondrial matrix by the adenine nucleotide translocator (ANT) (10,11). The dimeric cytosolic enzyme isoforms (Cy-CK) are also expressed in a quasi tissue specific fashion (7), consisting of the brain (BB-CK), the skeletal muscle (MM-CK) and the cardiac muscle heterodimer (MB-CK) (7,10).

Furthermore, because of the central role of the phosphocreatine/CK system in the regulation of brain ATP levels, alterations in CK have been observed in the brain in various pathologies of the central nervous system with altered energy metabolism, such as epilepsy, schizophrenia, Alzheimer's and Pick's disease (12-14). The decline in CK activity may reflect the severe disturbance in energy metabolism in the brain of these patients (14). Furthermore, knockout mice single mutants lacking B-CK as well as double mutants lacking both Mi_a-CK and B-CK display a number of notable abnormalities, such as decreased weight gain, reduced life expectancy, disturbed fat metabolism, impaired thermoregulation, resulting in sudden drops in body temperature and behavioral abnormalities (15).

In a previous study we have demonstrated that DGA significantly decreases CK activity from cerebral cortex, a brain structure manly affected in DHGA (16). In the present study, we tested the *in vitro* effects of DGA on total CK (tCK), Cy-CK and Mi-CK activities in the cerebellum of young rats in order to test the tissue specificity for DGA action and to better understand the pathogenetic mechanisms of brain injury of DHGA. We also examined the influence of the antioxidant glutathione (GSH), the free radical scavengers ascorbic acid and α-tocopherol (trolox), and the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) on the effects produced by DGA on CK activity since the enzyme contains thiol groups which are critical to its function and susceptible to oxidative stress. We observed that only Mi-CK activity was inhibited by DGA and that this inhibition was much weaker than that observed in cerebral cortex, only occurring with DGA doses 10-fold higher.

EXPERIMENTAL PROCEDURE

Subjects and Reagents. Wistar rats obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, were housed in groups of eight with their mothers on the day of birth. They were maintained on a 12:12 h light/dark cycle (lights on 07.00-19:00 h) in an air-conditioned constant temperature ($22°C \pm 1°C$) colony room, with free access to water and 20% (w/w) protein commercial chow

(Germani, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre and followed the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985).

All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA. D-2-hydroxyglutaric acid (DGA) was dissolved in Tris-HCl buffer, pH 7.5, for the enzymatic technique.

Preparation of total homogenates and the mitochondrial and cytosolic fractions. For the preparation of total homogenates, 30-day-old Wistar rats were sacrificed by decapitation without anaesthesia, the brain was rapidly removed and placed on a Petri dish on ice. The cerebellum was dissected and homogenized in 10 volumes of saline solution, with pH adjusted to 7.5 with NaOH, using a ground glass type Potter-Elvejhem homogenizer immersed in an iced box. At least two homogenizations of 30-s duration were performed at approximately 1000 ms/min with an electrically driven Teflon pestle. This homogenate was used for the estimation of total CK (tCK) activity. Mitochondrial and cytosolic fractions were prepared according to Ramirez and Jiménez (17). The cerebellum homogenate was centrifuged at 800 x g for 10 min at 4 °C and the pellet discarded. The supernatant was then centrifuged at 27 000 x g for 30 min at 4ºC in a Sorval DC-2B centrifuge. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the enzymatic assay (Mi-CK). The supernatant was further centrifuged at 125 000 x g for 60 min at 4ºC in a OTD-65B Sorval centrifuge, the microsomal pellet discarded, and the cytosol (supernatant) was used for the enzymatic assay (Cy-CK). The cytosolic fraction did not contain contaminating Mi-CK. In some experiments we used synaptic plasma membranes isolated from cerebral cortex (18) in order to determine Na^+,K^+–ATPase activity (19). The samples were stored at $– 40°$ C and the maximal period between total homogenate, sub-cellular preparations and enzyme analysis was always less than 5 days. The protein concentration in the preparations varied from 4 (cytosolic and mitochondrial fractions) to 12 mg (homogenates) protein . mL^{-1}.

Creatine Kinase (CK) Activities. CK activity was measured in total homogenates, as well as in the cytosolic and mitochondrial preparations from cerebellum. The reaction mixture contained the following final concentrations: 60 mM Tris-HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, and approximately 0.4 – 1.2 μg protein in a final volume of 0.1 mL. For enzymatic analysis in mitochondrial fractions, 0.625 mM lauryl maltoside was added to the medium. DGA, at 0.25 to 5 mM final
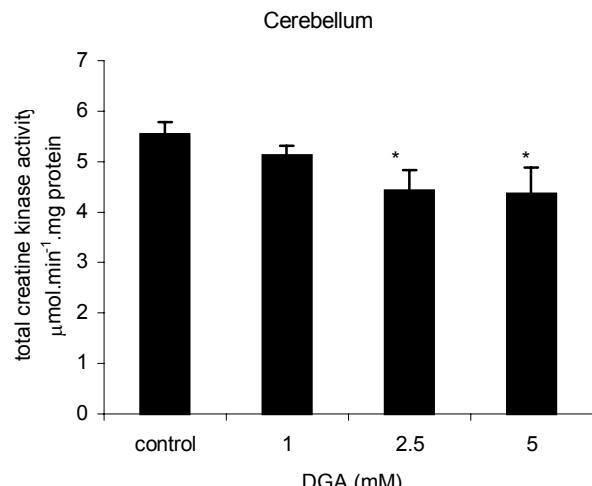
Cerebellum

Fig. 1. Effect of D-2-hydroxyglutaric acid (DGA) on total creatine kinase activity (μmol creatine / min . mg protein) in cerebellum homogenates of 30-day-old rats. Values are means $\pm$ SD for n = 5 independent experiments per group of creatine kinase activity measured in cerebellum homogenates in the presence or absence of the metabolite. One-way ANOVA [$F(3,19)=11.96$, p<0.0001] followed by the Duncan´s multiple range test (** p< 0.01 compared to control)

concentrations, was also supplemented to the medium, whereas controls did not contain the acid. After 15 minutes of pre-incubation at 37°C, the reaction was started by the addition of 3.2 mM ADP plus 0.8 mM reduced glutathione. The reaction was stopped after 10 minutes by the addition of 1 μmol p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls containing all components of the incubation medium except homogenates, were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (20). The color was developed by the addition of 0.1 mL 2 % α-naphthol and 0.1 mL 0.05 % diacetyl in a final volume of 1 mL and read at 540 nm after 20 minutes. In some experiments, homogenates were pre-incubated with 0.5 mM GSH, 1 mM L-NAME, 1 mM ascorbic acid or 10 μM trolox final concentrations for 15 min alone or in the presence of DGA in 60 mM Tris-HCl buffer, pH 7.5, containing 7 mM phosphocreatine and 9 mM $MgSO_4$, after which the other reagents were supplemented and reaction carried out. None of the substances supplemented to the assay interfered with the color development or spectrophotometer reading. Results were expressed as μmol of creatine formed per min per mg protein.
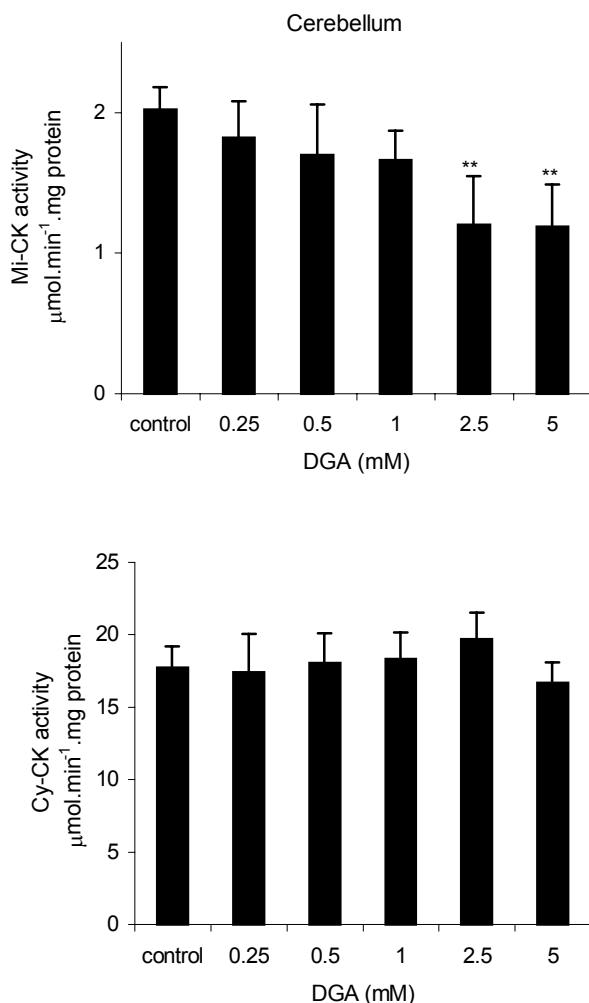
Fig. 2. Effect of D-2-hydroxyglutaric acid (DGA) on the mitochondrial creatine kinase (Mi-CK) and cytosolic (Cy-CK) activities (μmol creatine / min . mg protein) from cerebellum of 30-day-old rats. Values are means ± SD for n = 4-6 independent experiments per group in the presence or absence of the metabolite. One-way ANOVA [$F_{(5,23)}$=5.49; p=0.003] [$F_{(5,35)}$=0.545, p=0.741] followed by the Duncan´s multiple range test (** p< 0.01 compared to control).

Protein determination. Protein was measured by the method of Lowry et al. (21) using bovine serum albumin as standard.

Statistical analysis. Unless otherwise stated, results are presented as means ± standard deviation. All assays were performed in duplicate and the mean was used for the calculations. Data from the enzymatic activities were analyzed by one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. Dose-dependent effects were tested by regression analysis. Differences between the groups were rated significant at p < 0.05. All analyses were carried out in an IBM-compatible PC using the Statistical Package for the Social Sciences (SPSS) software.

RESULTS

We first examined the effect of DGA on total creatine kinase (tCK) activity in whole homogenates from cerebellum. Values for these activities in controls were similar to those described previously (10). It can be seen that DGA significantly inhibited (22% inhibition) tCK activity from cerebellum at concentrations of 2.5 mM and higher (Figure 1). We also examined the effect of DGA on Na^+,K^+-ATPase activity in synaptic plasma membrane from rat cerebellum, and observed that the metabolite did not affect this activity (control: 1,587 ± 289 μmol Pi.min^{-1}.mg protein; DGA: 1,314 ± 303 μmol Pi.min^{-1}.mg protein, n=5) [t(4)= 1,895 , p> 0.05].

We then investigated the effect of DGA on CK activity from the mitochondrial (Mi-CK) and cytosolic (Cy-CK) fractions of rat cerebellum. It can be seen that the acid significantly inhibited Mi-CK activity at 2.5 and greater concentrations (40% inhibition) (Figure 2A) but had no effect on Cy-CK activity (Figure 2B).

Next we determined the kinetics of the interaction of DGA with CK (22). The Lineweaver-Burk double-reciprocal plot was analyzed over a range of phosphocreatine concentrations (0.5 to 2.0 mM) in the absence or presence of DGA (1 to 5 mM). The data indicate that the inhibition of CK activity by DGA is non-competitive (Figure 3). The Km calculated was 2.43 mM ± 0.54 (n=3). The Ki value (the dissociation constant of the enzyme-substrate-inhibitor complex) was calculated by the method of Dixon (23), which provides a simple way of determining the inhibition constant (Ki) for non-competitive inhibitors. The Ki value calculated was 10.06 ± 2.77 mM (n= 3) for DGA.

Since CK is sensitive to oxidative modification (24) decreasing its activity after exposure to agents promoting generation of free radicals (25), we tested whether the significant reduction of cerebellum tCK activity caused by DGA could be mediated by oxidation of critical (–SH) groups on the enzyme. We therefore preincubated cerebellum homogenates in the presence of various antioxidants, the enzyme activity being measured afterwards. The concentrations of the antioxidants utilized in these assays were similar to those used by other investigators (26-28). We first observed that the nitric oxide (NO) synthase inhibitor L-NAME (1 mM) per se had no effect on CK activity, in contrast to ascorbic acid (1 mM) and DGA (5 mM), which significantly inhibited this enzyme activity. Furthermore, ascorbic acid and L-
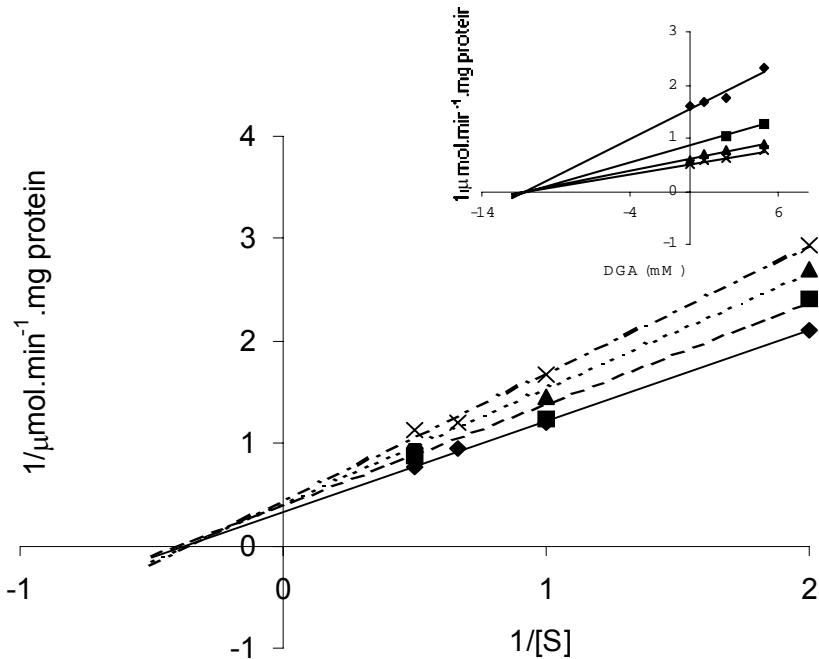
Fig. 3. Kinetic analysis of the inhibition of total creatine kinase (tCK) from cerebellum of rats by D-2-hydroxyglutaric acid. The graph shows a double reciprocal plot of creatine kinase for phosphocreatine concentrations (0.5-2.0 mM) [S] in the absence (♦ = controls) and in the presence of 1.0 (■), 2.5 (▲) and 5.0 (x) mM D-2-hydroxyglutaric acid. The inset shows the Dixon plot for Ki determination. All experiments were repeated at least three times and similar results were obtained. Data presented are representative of one experiment.

NAME did not prevent the inhibitory effect of DGA on the enzyme (Figure 4). We also tested the effects of the naturally-occurring antioxidant agent glutathione (0.5 mM GSH) and the free radical scavenger α-tocopherol (10 μM) on CK activity, and verified that these drugs per se did not affect tCK activity, but GSH fully prevented the inhibitory effect of DGA (Figure 5).

DISCUSSION

Some data in the literature indicate that mitochondrial dysfunction might be involved in the pathogenetic mechanisms of D-2-hydroxyglutaric aciduria (DHGA). In this context, it has been observed that affected patients excrete large quantities of Krebs cycle intermediates (2-4) and that DGA decreases glucose uptake and CO2 formation and inhibits the respiratory chain at various complexes in the cerebral cortex (6,29). In addition, DGA was recently postulated as an endogenous excitotoxic organic acid acting through NMDA receptors and resulting in cell death in cultured neurons from embryo chick telencephalon and neonatal rat hippocampus (29). These investigators also found that DGA induced the generation of reactive oxygen species (ROS) measured by the oxidant-sensitive dye dihydrorhodamine-123 in chick neurons, which was partially prevented by the NMDA glutamate receptor antagonist MK-801. Therefore, it is

possible that free radical production occurred, at least in part, secondarily to NMDA overstimulation. Despite these studies, the specific pathogenetic mechanisms of DGA on the central nervous system are still not fully established.

In a previous study, we have demonstrated that DGA significantly inhibits total CK activity in cerebral cortex, as well as the mitochondrial and cytosolic fractions, at concentrations as low as 0.25 mM, being the mitochondrial isoform more affected (16). In the present study, we investigated the *in vitro* effects of DGA on CK activities from cerebellum of young rats in the hope to determine whether the inhibitory effect of DGA on CK was tissue specific, and also to verify whether oxidative damage could be responsible for the inhibitory effect of this organic acid. Maintenance of this enzyme activity is critical for brain normal function and reduction of it may lead to selective tissue damage (8,30-32).

We initially verified that DGA significantly reduced total creatine kinase activity from the cerebellum of the animals at only 2.5 mM and higher concentrations. The kinetic analysis performed by the Lineweaver-Burk double-reciprocal plot revealed that this inhibition was noncompetitive. Furthermore, we demonstrated that DGA strongly inhibited the mitochondrial isoform of the enzyme (Mi-CK), but did not affect the cytosolic CK activity (Cy-CK). It is therefore possible that DGA effect was directed towards the mitochondrial isoform of the
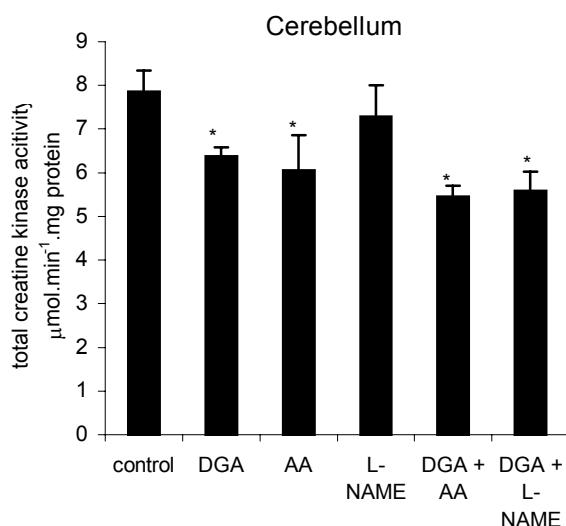
Fig. 4- Effect of ascorbic acid (AA) and L-NAME on cerebellum tCK activity in the presence or absence of D-2-hydroxyglutaric acid (DGA). Cerebellum homogenate preparations were pre-incubated at 37 °C for 15 min with 5.0 mM DGA, 1 mM AA, 5 mM DGA plus 1 mM AA, 1 mM L-NAME, or 5.0 mM DGA plus 1 mM L-NAME, and the enzyme activity was determined afterwards. Data are means $\pm$ SD for 6 independent experiments. One-way ANOVA [F(5,29)=15.19, p < 0.0001] followed by the Duncan´s multiple range test (* p < 0.05 compared to control).

enzyme Mi_a-CK, which is predominantly present in the brain.

The present study shows that cerebellum was much less vulnerable to the DGA-induced inhibitory effect than cerebral cortex since 0.25 mM DGA was already inhibitory in the cerebral cortex, as compared to the cerebellum, where this inhibition was achieved only with 10-fold higher (2.5 mM) DGA concentrations. This is in agreement with other studies showing that other neurotoxic agents provoke a greater *in vitro* and *in vivo* inhibition towards CK activity in cerebral cortex or/and other cerebral structures than in cerebellum (33,34). The exact mechanisms underlying these findings are still unknown, but may be related to the distinct proportions of the various CK isoforms in the cerebellum and in other cerebral structures (35-37).

In this context, it should be emphasized that brain structural abnormalities of patients affected by DHGA are mainly localized in the cerebral cortex, with practically no apparent injury in the cerebellum (2,3,38). Taken together, it may be presumed that the differential inhibition of this important enzyme activity necessary to maintain brain energy homeostasis may potentially explain, at least in part, the neuropathology of DHGA. However, we cannot rule out other important factors to explain the pathological findings in the brain of these individuals.

An interesting aspect of our previous study using cerebral cortex was that, although Mi-CK activity was more susceptible to DGA action than Cy-CK activity, both activities were inhibited (16). In the present study we

could not detect a significant inhibition for the cytosolic activity, in contrast with the mitochondrial CK, which was significantly inhibited by the acid. We cannot establish at the present time the reason for these results, but they may be due to distinct isoforms of the enzyme in various brain structures (35). In addition, it has been demonstrated different cytosolic (B-CK) subspecies in the brain, called Ba-CK and Bb-CK, which were shown to dimerize in a tissue-specific fashion (39). Therefore, because the differential tissue specificity of these Cy-CK subspecies, it may be presumed that DGA could have acted selectively on the distinct cerebral structures examined, i.e., inhibiting Cy-CK activity only in the cerebral cortex.

CK possesses eight sulfhydryl groups, and several reagents acting on these groups are able to inactivate the enzyme (40). There is a highly conserved cysteine reside located near the catalytic site of CK, which is crucial for full enzyme activity (41). It probably interacts with the guanidine group of the creatine substrate and is most likely responsible for its correct orientation within the active site (42). This cysteine residue was identified as the main target for chemical inactivation of CK by a large number of reagents (43,44). This inactivation is however partly reversible by reducing agents acting on the enzyme thiol groups (43-45). In addition, It has been also demonstrated that CK is sensitive to oxidative modification (24) and that its activity decreases after exposing brain to agents promoting free radical generation (25). NO, peroxynitrite and other free radicals inactivate the enzyme in skeletal muscle (46) and also in cardiac muscle (47-50).
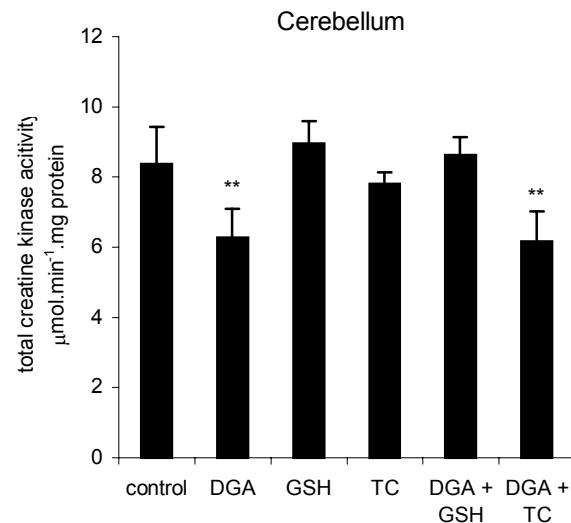
Fig. 5- Effect of α-tocopherol (TC) and reduced glutathione (GSH) on cerebellum tCK activity in the presence or absence of D-2-hydroxyglutaric acid (DGA). Cerebellum homogenates were pre-incubated at 37 °C for 15 min with 5.0 mM DGA, 10 μM TC, 5.0 mM DGA plus 10 μM TC, 0,5 mM GSH, or 5.0 mM DGA plus 0,5 mM GSH, and the enzyme activity was determined afterwards. Data are means $\pm$ SD for 6 independent experiments. One-way ANOVA [F(5,35)=15.5, p<0.001] followed by the Duncan´s multiple range test (**p < 0.01 compared to control).

Therefore, we tested the influence of various antioxidants on the inhibitory effect of DGA on CK. We verified that GSH, that acts as a naturally-occurring thiol-reducing agent (51), fully prevented the inhibitory role of DGA on brain CK activity. Therefore, it is likely that this inhibition may be mediated by oxidation of sulfhydryl or other groups of the enzyme. These results are in accord with other studies showing that the free radical-induced inhibition of cardiac mitochondrial CK can be reversed *in vitro* by the reducing agents dithiothreitol (31,47) and 2-mercaptoethanol (49) and that ROS are generated by DGA in cultured neuronal cells (29). Furthermore, the strong inhibitory action of the neurotoxic gas methyl bromide on CK brain activity was shown to be prevented by the reducing agent dithiothreitol, indicating that thiol groups were involved in such inhibition, similarly to what occurred in our study (33).

It is interesting to note that ascorbic acid *per se* had a mild but significant inhibitory influence on the enzyme activity, a fact that was reported before in other systems and seems to be related to the ability of ascorbic acid, despite its known reducing action, to form ROS in the presence of traces of transition metal ions in the reaction solutions and to induce lipid peroxidation and other free radical damage, including protein inactivation, when combined with iron (52-53). In this context, it is well demonstrated that iron is found in high concentrations in the brain. We do not know the exact reasons why ascorbic acid inhibited CK activity in cerebellum, whereas the same did not occur in the cerebral cortex (16). However, it is possible that this effect occurred because of the distinct iron concentrations in the various brain regions (54), once ascorbic acid in the presence of iron can induce free radicals (52).

Although to our knowledge nothing is reported on the role of the redox potential on the activity of brain CK, our present findings are indicative that DGA possibly acts through oxidation or other alterations of critical thiol or other groups present in the enzyme molecule and necessary for its function. Furthermore, it can be also concluded that, under our *in vitro* assay conditions, NO or other common free radicals are not involved in the inhibitory effect of DGA in brain CK activity, since we used high doses of the NO synthase inhibitor L-NAME (1 mM) and the free radical scavengers ascorbic acid (1 mM) and α-tocopherol (10 μM), which did not prevent DGA inhibitory effect.

It is interesting to note that SH-groups play an important role for the enzyme CK in the region of dimer contacts and association in the octameric form, which is important for its *in vivo* function (55). It is thought that only the octameric Mi-CK is able to interact simultaneously with two opposing membranes, forming complexes with ANT and porin in the so-called mitochondrial contact sites between the outer and the inner mitochondrial membranes. These contact sites are important for efficient energy export into the cytosol by direct channeling of substrates and products in between

the interaction partners (56). Destabilization of the Mi-CK octamer leads to an impairment of Mi-CK function (57). Furthermore, the octameric state of Mi-CK is essential for its protective effect on the opening of the mitochondrial permeability transition pore (56). Therefore, our findings may lead one to speculate that DGA, by inhibiting the Mi-CK activity probably by oxidation of thiol groups, may possibly disrupt the octameric CK isoform, which is the most active.

At this point it should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues (58), a fact that makes this tissue more vulnerable to oxidation.

On the other hand, the precise mechanisms by which DGA suppresses Mi-CK activity, as demonstrated in the present study, are yet unclear, although oxidation or other alterations of important groups could inactivate the enzyme. Further studies will be required to investigate if a direct effect of this organic acid on the enzyme occurs and also whether the *in vitro* inhibition here observed can be also demonstrated *in vivo*.

It is interesting to note that DGA did not affect cerebellum Na^+,K^+ –ATPase activity, another enzyme containing essential thiol groups, as well as various respiratory chain activities in cerebral cortex, as previously demonstrated (16). These data indicate a rather selective action for DGA.

In summary, we demonstrated that DGA, a metabolite that characteristically accumulates in DHGA, moderately reduces the Mi-CK activity from cerebellum at concentrations of 2.5 mM and greater probably by oxidation or other modification of fundamental groups on the enzyme. Considering that the blood circulating and CSF levels of DGA in patients affected by DHGA (2,3) are about 46-757 μmol/L, it is difficult to envisage a significant inhibition of this enzyme activity occurring *in vivo* in the cerebellum of DHGA patients. However, since the brain concentrations of DGA in the affected individuals are yet unknown, we cannot exclude that higher intracerebral concentrations of DGA may be attained in this neurometabolic disease, as possibly occurs in other similar organic acidemias so-called "cerebral" organic acidemias whose symptoms are predominantly or almost exclusively neurological (59). Previous results from our laboratory demonstrated a marked inhibitory activities of DGA at concentrations as low as 0.25 mM in the cerebral cortex (16). Taken together, it is feasible that the differential inhibitory activities detected in the cerebral cortex and cerebellum may explain, at least in part, the neuropathological findings found in DHGA patients characterized by cerebral cortex atrophy with no apparent cerebellum injury.

Finally, compounds such as those that either buffer intracellular energy stores (creatine) or increase overall mitochondrial function and/or antioxidants (coenzyme Q_{10}) may hold a possible therapy for DHGA, as they possibly represent a great promise as an adjunct therapy for other neurodegenerative disorders (60,61).

REFERENCES

1. Chalmers, R. A., Lawson, A. M., Watts, R. W. E., Tavill, A. S., Kamerling, J. P., Hey E., and Ogilvie D. 1980. D-2-Hydroxyglutaric aciduria: case report and biochemical studies. J. Inherit. Metab. Dis. 3:11-15.

2. van der Knaap, M. S., Jakobs, C., Hoffman, G. F., Duran, M., Muntau, A. C., Schweitzer, S., Kelley, R. I., Parrot-Rouland, F., Amiel, J., De Lonlay, P., Rabier, D., Eeg-Olofsson, O. 1999. D-2-Hydroxyglutaric aciduria: further clinical delineation. J. Inherit. Metab. Dis. 22:404-413.

3. van der Knaap, M. S., Jakobs, C., Hoffmann, G. F., Nyhan, W. L., Renier, W. O., Smeitink, J. A. M., Catsman-Berrevoets, C. E., Hjalmarson, O., Vallance, H., Sugita, K., Bowe, C. M., Herrin, J. T., Craigen, W. J., Buist, N. R. M., Brookfield, D. S. K., Chalmers, R. A. 1999. D-2-Hydroxyglutaric aciduria: biochemical marker or clinical disease entity? Ann. Neurol. 45:111-119.

4. Wajner, M., Vargas, C.R., Funayama, C. et al. 2002. D-2-hydroxyglutaric aciduria in a patient with a severe clinical phenotype and unusual MRI findings. J. Inherit. Metab. Dis. 25:28-34.

5. Gibson, K.M., Craigen, W., Herman, G.E., Jakobs, C. 1993. D-2-hydroxyglutaric aciduria in a newborn with neurological abnormalities: a new neurometabolic disorder? J. Inherit. Metab. Dis. 16:497-500.

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

7. Wallimann, T., Wyss, M., Brdiczka, D., and Nicolay, K. 1992. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem. J. 281:21-40.

8. Wyss, M., Smeitink, J., Wevers, R. A., and Wallimann, T. 1992. Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. Biochim. Biophys. Acta, 1102:119-166.

9. Schlegel, J., Zurbriggen, B., Wegmann, G., Wyss, M., Eppenberger, H., and Wallimann, T. 1988. Native mitochondrial creatine kinase forms octameric structures. I. Isolation of two interconvertible mitochondrial creatine kinase forms, dimeric and octameric mitochondrial creatine kinase: Characterization, localization and structure-function relationships. J. Biol. Chem. 262:16942-16993.

10. O'Gorman, E., Beutner, G., Wallimann, T., and Brdiczka, D. 1996. Differential effects of creatine depletion on the regulation of enzyme activities and on creatine-stimulated mitochondrial respiration in skeletal muscle. Biochim. Biophys. Acta 1276:161-170.

11. Saks, V.A., Kuznetsov, A.V., Kuprianov, V.V., Miceli, M.V., and Jacobus, W.E. 1985. Creatine kinase of rat heart mitochondria. The demonstration of functional coupling to oxidative phosphorylation in an inner membrane-matrix preparation. J. Biol. Chem. 260:7757-7764.

12. Hemmer, W., and Wallimann, T. 1993. Functional aspects of creatine kinase in brain. Dev. Neurosci. 15:249-260.

13. Burbaeva, G.S., Aksenova, M.V., and Makarenko, I.G. 1992. Decrease level of creatine kinase BB in the frontal cortex of Alzheimer patients. Dementia 3: 91-94.

14. Aksenov, M.I., Aksenova, M.V., Payne, R.M., Smith, C.D., Markesbery, W.R., and Carney, J.M. 1997. The expression of creatine kinase isoenzymes in neocortex of patients with neurodegenerative disorders: Alzheimer's and Pick's disease. Exp. Neurol. 146: 458-465.

15. Wyss, M. and Shulze, A. 2002. Health imlicatinos of creatine: can oral creatine supplementation protect against neurological and atherosclerotic disease? Neuroscience, 112:243-260.

16. Silva, C.G., Bueno, A.R.F., Schuck, P.F., Leipnitz, G., Ribeiro, C.J.A., Rosa, R.B., Dutra Filho, C.S., Wyse, A.T.S., Wannmacher, C.M.D., and Wajner, M. (submitted 2003) Inhibition of creatine kinase activity from rat cerebral cortex by D-2-hydroxyglutaric acid in vitro. Neurochem. Int.

17. Ramirez, O., and Jiménez, E. 2000. Opposite transitions of chick brain catalytically active cytosolic creatine kinase isoenzymes during development. Int. J. Dev. Neurosci. 18:815–823.

18. Jones, D.H., Matus, A.I. 1974. Isolation of plasma synaptic membrane from brain by combination flotation–sedimentation density gradient centrifugation. Biochim. Biophys. Acta 356: 276–287.

19. Tsakiris, S., Deliconstantinos, G. 1984. Influence of phosphatidylserine on (Na^+, K^+)-stimulated ATPase and acetylcholinesterase activities of dog brain synaptossomal plasma membranes. J. Biochem. 22: 301–307.

20. Hughes, B. P. 1962. A method for estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin. Chim. Acta 7:597-604.

21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-267.

22. Cornish-Bowden, A. 1974. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem. J. 137:143-144.

23. Dixon, M,. and Webb, E. C. 1964. Enzymes, Longman, London, 2nd Edition.

24. Oliver, C.N., Starke-Reed, P.E., Stadtman, R., Liu, G.J., Carney, J.M. and Fliyd, R.A. 1990. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion injury to gerbil brain. Proc. Natl. Acad. Sci. 87:5144-5147.

25. Burmistrov, S.O., Mashek, O.P., and Kotin, A.M. 1992. The action of acute alcoholic intoxication on the antioxidant system and creatine kinase activity in the brain of rat embryos. Eksp. Klin. Farmakol. 55: 54-56.

26. Stanimirovic, D.B., Wong, J., Ball, R., Durkin, J.P. 1995. Free radical-induced endothelial membrane dysfunction at the site of blood-brain barrier: relationship between lipid peroxidation, Na,K-ATPase activity, and 51Cr release. Neurochem. Res. 20: 1417-1427.

27. Avrova, N.F., Victorov, I.V., Tyurin, V.A., Zakharova, I.O., Sokolova, T.V., Andreeva, N.A., Stelmaschuk, E.V., Tyurina, Y.Y., Gonchar, V.S. 1998. Inhibition of glutamate-induced intensification of free radical reactions by gangliosides: possible role in their protective effect in rat cerebellar granule cells and brain synaptosomes. Neurochem. Res. 23: 945-952.

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

29. Kölker, S., Pawlak, V., Ahlemeyer, B., Okun, J.G., Hörster, F., Mayatepek, E., Krieglstein, J., Hoffman, G.F., and Köhr, G. 2002. NMDA receptor activation and respiratory chain complex V inhibition contribute to neurodegeneration in D-2-hydroxyglutaric aciduria. Eur. J. Neurosci. 16:21-28.

30. Hamman, B.L., Bittl, J.A., Jacobus, W.E., Aleen, P.D., Spencer, R.S., Tina, R., and Ingwall, J.S. 1995. Inhibition of creatine kinase reaction decrease the contractile reserve of isolated rat hearts. Am. J. Physiol. 269:H1030-H1036.

31. Gross, W. L., Bak, M. I., Ingwall, J. S., Arstal, l M. A., Smith, T. W., Balligand, J. L., and Kelly, R. 1996. Nitric oxide inhibits creatine kinase and regulates rat heart contractile reserve. Proc. Natl. Acad. Sci. U. S. A. 93:5604–5609.

32. Holzman, D., Meyers, R., Khait, I., and Jensen, F. 1997. Brain creatine kinase reaction rates and reactant concentrations during seizures in developing rats. Epilepsy Res. 27:7-11.

33. Hyakudo, T., Hori, H., Tanaka, I., Igisu, H. 2001. Inhibition of cretine kinase activity in rat brain by methyl bromide gas. Inhal. Toxicol. 13:659-669.

34. Matsuoka, M., Inoue, N., Igisu, H. 1992. Effects of methylmercury

chloride on creatine kinase activity in the rat brain. J. UOEH, 14:77-81.

35. Kaldis, P. Hemmer, W., Zanolla, E., Holtzman, D., Wallimann, T. 1996. 'Hot Spots' of creatine kinase localization in brain: cerebellum, hippocampus and choroid plexus. Dev. Neurosci. 18:542-554.

36. Ilyin, S.E., Sonti, G., Molloy, G., Plata-Salaman, C.R. 1996. Creatine kinase-B mRNA levels in brain regions from male and female rats. Brain Res. Mol. Brain Res. 41:50-56.

37. Hemmer, W., Zanolla, E., Furter-Graves, EM., Eppenberger, H.M., Wallimann, T. 1994. Creatine kinase isoenzymes in chicken cerebellum: specific localization of brain-type creatine kinase in Bergmann glial cells and muscle-type creatine kinase in Purkinje neurons. Eur. J. Neurosci. 6:538-549.

38. Eeg-Olofsson, O. 2000. D-2-hydroxyglutaric aciduria with cerebral, vascular and muscular abnormalities in a 14-year-old-boy. J.Child. Neurol. 15: 488-492.

39. Quest, A.F.G., Eppenberger, H.M., Wallimann, T. 1990. Two different B-type creatine kinase subunits dimerize in a tissue-specific manner. FEBS Lett. 2:299-304.

40. Kenyon, G.L., and Reed, G.H. 1983. Creatine kinase: structure-activity relationships. Adv. Enzymol. 54:367-426.

41. Furter, R., Furter-Graves, E.M., and Wallimann, T. 1993. Creatine kinase: the reactive cysteine is required for synergism but is nonessential for catalysis. Biochemistry 32:7022-7029.

42. Zhou, G., Somasyndaram, T., Blanc, E., Parthasarathy, G., Ellingtin, W.R., and Chapman, M.S. 1998. Transition state structure of arginine kinase: implications for catalysis of bimolecular reactions. Proc. Natl. Acad. Sci. USA 95:8449-8454.

43. Brown, C.S., and Cunningham, L.W. 1970. Reaction of reactive sulfhydryl groups of creatine kinase with dansyl chloride. Biochemistry 9: 3878-3885.

44. Reddy, S.R. and Watts, D.C. 1979. Inhibition of creatine kinase by iodoalkanes. Further appraisal of the essential nature of the reactive thiol group. Biochim. Biophys. Acta 569:109-113.

45. Hou, L.W., and Vollmer, S. 1994. The activity of S-thiomethyl modified creatine kinase is due to the regeneration of free thiol at the active site. Biochim. Biophys. Acta 1205:83-88.

46. Wolosker, H., Panizzutti, R. and Englender, S. 1996. Inhibition of creatine kinase by S-nitrosoglutathione. FEBS Lett. 392:274-276.

47. Mekhfi, H., Veksler, V., Mateo, P., Maupoil, V., Rochette, L., and Ventura- Clapier, R. 1996. Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils. Circ. Res. 78:1016-1027.

48. Stachowiak, O., Dolder, M., Wallimann, T., and Richter, C. 1998. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J. Biol. Chem. 273:16694-16699.

49. Nathan, C. and Xie, Q. W. 1994. Nitric oxide synthases: roles, tools, and controls. Cell 78:915-918.

50. Yuan, G., Kaneko, M., Masuda, H., Hon, R. B., Kobayashi, A. and Yamazaki, N. 1992. Decrease in heart mitochondrial creatine kinase activity due to oxygen free radicals. Biochim. Biophys. Acta. 1140:78-84

51. Meister, A., and Anderson, M. E. 1983. Glutathione. Annu. Rev. Biochem. 52:711-760.

52. Halliwell, B., and Gutteridge, J.M.C. 1990. Role of free radicals and catalytic metal ions in human disease. Methods Enzymol. 186:1-85.

53. Powers, S.K., and Hamilton, K. 1999. Anti-oxidants and exercise. Clin. Sports Med.18:525-536

54. Jane, A., Roskams, I., Connor, J.R. 1994. Iron, transferrin and ferritin in rat brain during development and aging. J. Neurochem. 63:709-716.

55. Lipskaya, T.Yu. 2001. Mitochondrial creatine kinase: Properties and function. Biochemistry (Mosc). 66:1361-1376.

56. Soboll, S., Brdiczka, D., Jahnke, D., Schmidt, A., Schlattner, U., Wendt, S., Wyss, M., and Wallimann, T. 1999. Octamer-dimer transitions of mitochondrial creatine kinase in hear disease. J. Mol. Cell Cardiol. 31:857-866.

57. Khuchua, Z.A., Qin, W., Boero, J., Cheng, J., Payne, R.M., Saks, V.A., and Strauss, A.W. 1998. Octamer formation and coupling of cardiac sarcomeric mitochondrial creatine kinase are mediated by charged N-terminal residues. J. Biol. Chem. 273:22990-22996.

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

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

60. Korosheltz, W.J., Jenkins, B.G., Rosen, B.R., and Beal, M.F. 1997. Energy metabolism defects in Huntington's disease and effects of coenzyme Q$_{10}$. Ann. Neurol. 41:120-130.

61. Shults, C.W., Beal, M.F., Fontaine, D., Nakano, K., and Haas, R.H. 1998. Absorption, tolerability, and effects of mitochondrial activity of oral coenzyme Q$_{10}$ in parkisonian patients. Neurology 50:793-795.

III. 5. Ácido L-2-hidroxiglutárico inibe a atividade da creatina quinase mitocondrial em cerebelo de ratos jovens

L-2-hydroxyglutaric acid inhibits mitochondrial creatine kinase activity from cerebellum of developing rats

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Abstract

L-2-Hydroxyglutaric acid (LGA) is the biochemical hallmark of patients affected by the neurometabolic disorder known as L-2-hydroxyglutaric aciduria (LHGA). Although this disorder is predominantly characterized by severe neurological findings and pronounced cerebellum atrophy, the neurotoxic mechanisms of brain injury are virtually unknown. In the present study, we investigated the effect of LGA, at 0.25–5 mM concentrations, on total creatine kinase (tCK) activity from cerebellum, cerebral cortex, cardiac muscle and skeletal muscle homogenates of 30-day-old Wistar rats. CK activity was measured also in the cytosolic (Cy-CK) and mitochondrial (Mi-CK) fractions from cerebellum. We verified that tCK activity was significantly inhibited by LGA in the cerebellum, but not in cerebral cortex, cardiac muscle and skeletal muscle. Furthermore, CK activity from the mitochondrial fraction was inhibited by LGA, whereas that from the cytosolic fraction of cerebellum was not affected by the acid. Kinetic studies revealed that the inhibitory effect of LGA on Mi-CK was non-competitive in relation to phosphocreatine. Finally, we verified that the inhibitory effect of LGA on tCK was fully prevented by pre-incubation of the homogenates with reduced glutathione (GSH), suggesting that this inhibition is possibly mediated by oxidation of essential thiol groups of the enzyme. Considering the importance of creatine kinase activity for energy homeostasis, our results suggest that the selective inhibition of this enzyme activity by increased levels of LGA could be possibly related to the cerebellar degeneration characteristically found in patients affected by L-2-hydroxyglutaric aciduria.
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Keywords: L-2-Hydroxylgutaric acid; Brain metabolism; Creatine kinase

1. Introduction

Creatine kinases (CKs, EC 2.7.3.2) comprehend a family of enzymes catalyzing the reversible transfer of a phosphoryl group between ATP and creatine (Bessman, 1985). CKs are present in high quantities in tissues that have high-energy flux or requirements such as skeletal muscle, cardiac muscle and brain (Schlegel et al., 1988). They are formed by a group of isoenzymes consisting of the cytosolic dimers BB-CK (brain), MM-CK (muscle) and the heterodimer MB-CK, which is present only in cardiac muscle cytosol. In addition, there are two mitochondrial-specific CKs (Mi-CK) posi-

tioned in the intermembrane space, termed Mib-CK (specific for sarcomeric muscle) and Mia-CK (the ubiquitous isoform) (Wallimann et al., 1992). A functional interplay between mitochondrial and cytosolic isoforms of CK is thought to be important for the regulation of cellular energy homeostasis. The cytosolic isoenzymes re-phosphorylate the locally produced free ADP from phophocreatine, releasing ATP and creatine. The mitochondrial enzyme catalyses the reaction of creatine to phosphocreatine at the expense of mitochondrial ATP (Wallimann et al., 1992; Wyss et al., 1992; Bessman and Carpenter, 1985; Jacobus, 1985). Because of the central role of the phosphocreatine/CK system in the regulation of brain ATP levels, alterations in CK activity have been proposed to explain the neuropathology of some degenerative disorders of the CNS presenting altered energy metabolism (Hemmer and Walliman, 1993). Furthermore, impaired CK function seems to be of significance for the development of various pathological states including myocardial diseases (Hamman et al., 1995; Gross et al., 1996; Liao et al., 1996). L-2-Hydroxyglutaric aciduria (LHGA) is a rare organic

aciduria, first described in 1980 by Duran et al. (1980). It has now been identified in approximately 50 patients. The clinical phenotype is variable, including cerebellar ataxia, choreiform movements, seizures, macrocephaly, speech and mental retardation and developmental neurodegeneration presenting in infancy and childhood (Barth et al., 1992). Furthermore, two other forms of LHGA have also been described, one more severe presenting with hypotonia, apnoea and convulsions shortly after birth (Chen et al., 1996) and another affecting older individuals (Fujitake et al., 1999).

Cerebellar symptoms and mental deficiency are present in almost all patients. The magnetic resonance imaging (MRI) pattern typically shows subcortical white matter loss, cerebellar atrophy, changes in dentate nuclei and putamen and subcortical leukoencephalopathy (Barth et al., 1992, 1993; Chen et al., 1996; Fujitake et al., 1999; Barbot et al., 1997). Biochemically, this disorder is characterized by increased tissue accumulation and urinary excretion of L-2-hydroxyglutaric acid (Barth et al., 1992). Besides the characteristic high levels of LGA in plasma, CSF and urine, a few affected patients present increased concentrations of lactate and/or Krebs cycle intermediates in their biological fluids (Hoffmann et al., 1995; Barth et al., 1998).

The metabolic block involved in LHGA is so far unknown, but may involve an unidentified step in the lysine catabolism pathway since lysine is also increased in blood and CSF (Barth et al., 1992; Barbot et al., 1997; Hoffmann et al., 1995). Moreover, virtually nothing is known about the pathophysiology of the neurological dysfunction of LHGA. However, considering that elevated levels of lactate and Krebs cycle intermediates accumulate in patients affected by this disorder, it is conceivable that energy metabolism dysfunction may be associated with this disease.

Therefore, considering that CK plays an important role in brain energy homeostasis, in the present study we investigated the in vitro effects of LGA on CK activities in total homogenates creatine kinase (tCK) from cerebral cortex, cerebellum, skeletal and cardiac muscle, as well as on the cytosolic (Cy-CK) and mitochondrial fractions prepared from cerebellum of young rats, in the hope to clarify the pathogenetic mechanisms of tissue injury in LHGA. We also tested the influence of the antioxidant glutathione (GSH), the free radical scavengers ascorbic acid and α-tocopherol (TC) and the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) on the effects produced by LGA on CK activities since the enzyme contains thiol groups which can be oxidized and are critical to its function.

2. Materials and methods

2.1. Subjects and reagents

Wistar rats obtained from the Central Animal House of the Department of Biochemistry, Instituto de Ciências Bási-

cas da Saúde, Federal Universidade de Rio Grande do Sul, Porto Alegre, Brazil, were housed in groups of eight with their mothers on the day of birth. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in an air-conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and a 20% (w/w) protein commercial chow (Germani, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre and followed the "Principles of Laboratory Animal Care (NIH publication 85–23, revised 1985).

All chemicals were purchased from Sigma, St. Louis, MO, USA. L-2-hydroxyglutaric acid was dissolved in Tris–HCL buffer, pH 7.5, for the enzymatic technique.

2.2. Preparation of total homogenates and the mitochondrial and cytosolic fractions

For the preparation of total homogenates, 30-day-old Wistar rats were sacrificed by decapitation, the brain was rapidly removed, pons and medulla were discarded. The cerebral cortex devoid of the deep white matter and cerebellum were dissected and homogenized in 10 vol. of saline solution, pH 7.5, using a ground glass type Potter–Elvejhem homogenizer immersed in an iced box. At least two homogenizations of 30 s duration were performed at approximately 1000 ms/min with an electrically driven Teflon pestle. The skeletal and cardiac muscles were also isolated and the homogenates were obtained as described above. These homogenates were used for the estimation of total CK (tCK) activity. For the preparation of mitochondrial and cytosolic fractions, the homogenates were centrifuged at $800 \times g$ for 10 min at 4 °C and the pellet discarded (Ramirez and Jiménez, 2000). The supernatant was then centrifuged at $27,000 \times g$ for 30 min at 4 °C in a Sorval DC-2B centrifuge. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the mitochondrial creatine kinase enzymatic assay. The supernatants were further centrifuged at $125,000 \times g$ for 60 min at 4 °C in a OTD-65B Sorval centrifuge, the microsomal pellet discarded, and the cytosol (supernantant) was used for the cytosolic creatine kinase enzymatic assay. The maximal period between total homogenate and subcellular preparations and enzyme analysis was always less than 5 days and these preparations were stored at −40 °C until enzymatic analysis. The protein concentration in the preparations varied from 4 (cytosolic and mitochondrial fractions) to 12 mg (homogenates) protein per ml.

2.3. Creatine kinase activities

CK activity was measured in total homogenates from cerebellum, cerebral cortex, cardiac and skeletal muscle, as well as in the cytosolic and mitochondrial preparations from

cerebellum. The reaction mixture consisted of the following medium: 60 mM Tris–HCl buffer, pH 7.5, containing 7 mM phosphocreatine, 9 mM MgSO$_4$, and approximately 0.4–1.2 μg protein in a final volume of 0.1 ml. For enzymatic analysis in mitochondrial fractions, 0.625 mM lauril maltoside was added to the incubation mixture. LGA at 0.25–5 mM final concentrations was also supplemented to the medium, whereas controls did not contain the acid. After 15 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol ADP plus 0.08 μmol reduced glutathione. The reaction was stopped after 10 min by the addition of 1 μmol p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 ml 2% α-naptol and 0.1 ml 0.05% diacetyl in a final volume of 1 ml and read after 20 min at 540 nm. In some experiments, the homogenates were pre-incubated with 0.5 mM glutathione, 1 mM L-NAME, 1 mM ascorbic acid or 10 μM α-tocopherol (trolox) final concentrations for 15 min alone or in the presence of the acid, after which the other reagents were supplemented and reaction carried out. None of the substances supplemented to the assay medium interfered with the color development or spectrophotometer reading. Results were expressed as μmol of creatine formed per min per mg protein.

2.4. Protein determination

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.
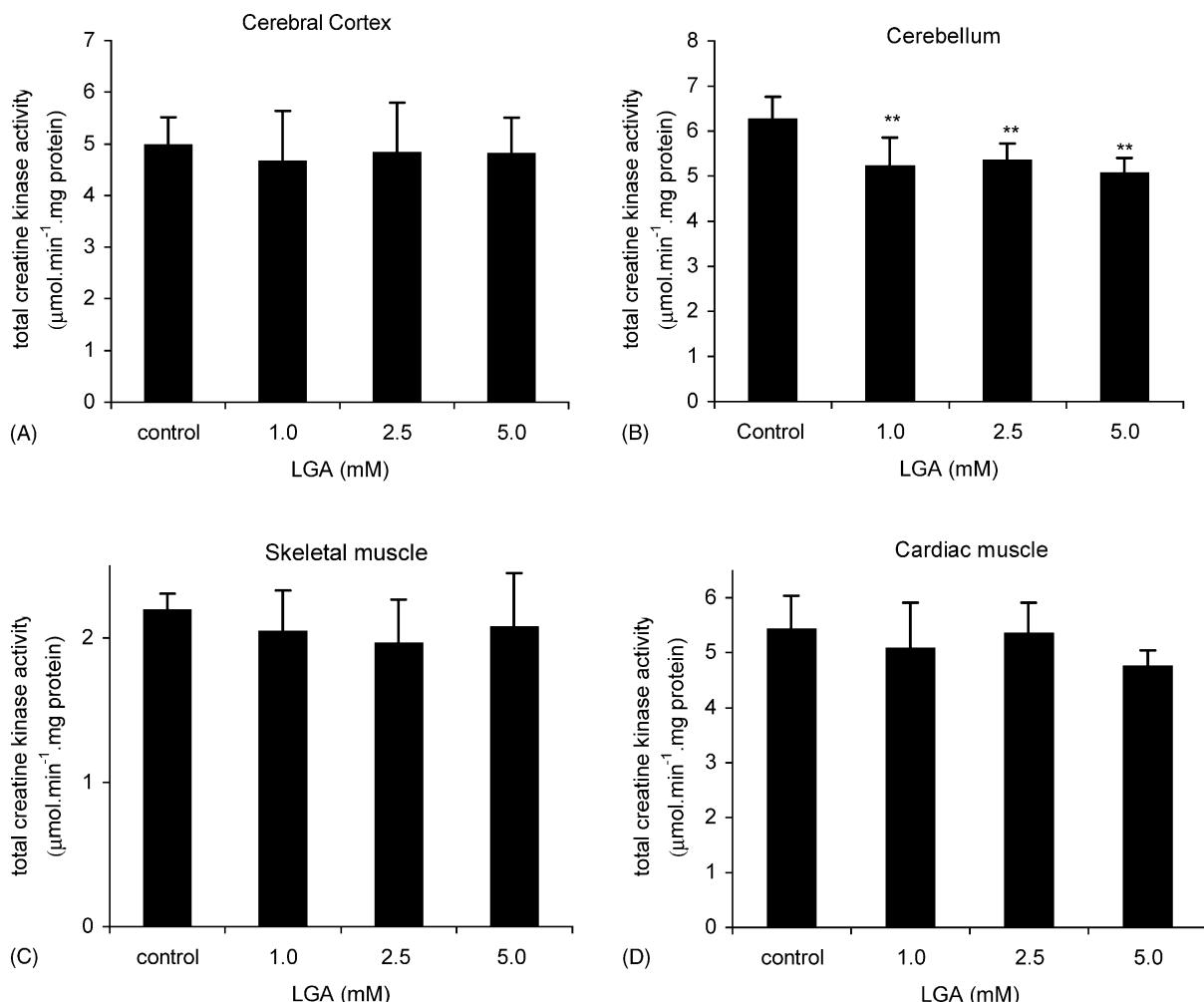
Fig. 1. Effect of L-2-hydroxyglutaric acid (LGA) on total creatine kinase activity (μmol creatine/min mg protein) from cerebral cortex (A), cerebellum (B), skeletal muscle (C) and cardiac muscle (D) homogenates of 30-day-old rats. Values are means ± S.D. for $n = 4$–7 independent experiments per group of creatine kinase activity measured in tissue homogenates in the presence or absence of the metabolite. ** $P < 0.01$ compared to control (one-way ANOVA followed by the Duncan's multiple range test).

2.5. Statistical analysis

Unless otherwise stated, results are presented as means ± standard deviation (S.D.). All assays were performed in duplicate and the mean was used for statistical analysis. Data from the activity of creatine kinase were analyzed using the one-way analysis of variance (ANOVA) followed by the post hoc Duncan's multiple range test when F was significant. For analysis of dose-dependent effect, linear regression was used. The Student's *t*-test was also used for comparison of two means. Differences between the 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 examined the effect of LGA, at concentrations varying from 1 to 5 mM, on CK activity in total homogenates from cerebellum, cerebral cortex, skeletal muscle and cardiac muscle. Values for these activities in controls were similar to those described previously (O'Gorman et al., 1996). LGA did not alter tCK activities from cerebral cortex ($F(3, 27) = 0.164$; $P = 0.92$), skeletal muscle ($F(3, 19) = 0.543$; $P = 0.66$), and cardiac muscle ($F(3, 23) = 1.465$; $P = 0.254$), but significantly inhibited the enzyme activity from cerebellum at all concentrations tested ($F(3, 23) = 7.152$; $P < 0.001$) (Fig. 1).

We then investigated the effect of LGA at concentrations varying from 0.25 to 5 mM on CK activity from the mitochondrial and cytosolic fractions of rat cerebellum. It can be seen that the metabolite significantly inhibited Mi-CK activity ($F(5, 23) = 2.699$; $P < 0.05$) in a dose-dependent fashion ($\beta = -0.640$, $P < 0.0001$) (Fig. 2A), but had no effect on Cy-CK activity ($F(5, 35) = 0.104$, $P = 0.991$) (Fig. 2B).

Next, we examined the kinetics of the interaction of LGA with Mi-CK from cerebellum (Cornish-Bowden, 1974). The Lineweaver–Burk double-reciprocal plot was analyzed over a range of creatine phophate concentrations (0.5–2.0 mM) in the absence or presence of LGA (1–5 mM). The data indicate that the inhibition of Mi-CK activity by LGA is non-competitive (Fig. 3). The Km calculated was 2.52 ± 0.74 mM ($n = 4$). The Ki value (the dissociation constant of the enzyme–substrate–inhibitor complex) was calculated by the method of (Dixon and Webb, 1964) which provides a simple way of determining the inhibition constant (Ki) for non-competitive inhibitors. The Ki value calculated was 11.13 ± 3.71 mM for LGA.

In order to test whether the significant reduction of cerebellum CK activity caused by LGA was mediated by oxidation of critical thiol (–SH) groups of the enzyme, we pre-incubated cerebellum homogenates in the presence of the antioxidant glutathione, the free radical scavengers
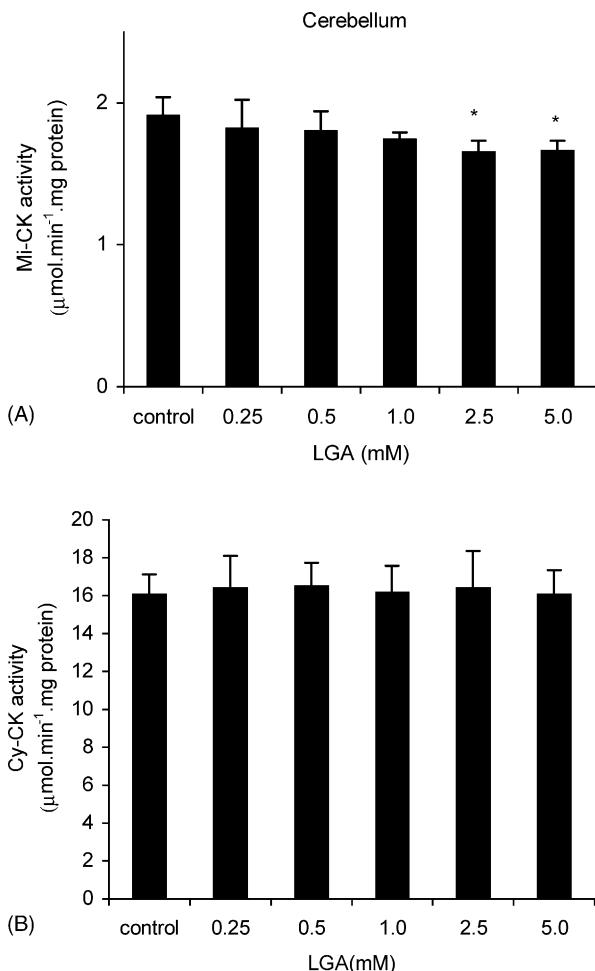
Fig. 2. (A and B) Effect of L-2-hydroxyglutaric acid on the mitochondrial (Mi-CK) and cytosolic creatine kinase (Cy-CK) activities (μmol creatine/min mg protein) in cerebellum preparations of 30-day-old rats. Values are means ± S.D. for $n = 4$–6 independent experiments per group of Mi-CK activity measured in the presence or absence of the metabolite. $^{*}P < 0.05$ compared to control (one-way ANOVA followed by the Duncan's multiple range test).

ascorbic acid and α-tocopherol or the nitric oxide synthase inhibitor L-NAME. The enzyme activity was measured in total cell homogenates since free radicals can be better produced with the whole cell machinery. First, by pre-incubating cerebellum homogenates in the presence of ascorbic acid (1 mM) or L-NAME (1 mM), alone or combined with 5 mM LGA, we observed that L-NAME per se had no effect on the on tCK activity, in contrast to ascorbic acid and LGA which significantly inhibited the enzyme activity. Furthermore, ascorbic acid and L-NAME did not prevent the inhibitory effect of LGA on this activity ($F(5, 47) = 11.31$, $P < 0.0001$) (Fig. 4). We also tested the effects of glutathione (0.5 mM GSH) and the free radical scavenger α-tocopherol (10 μM) on CK activity. We verified that these drugs per se did not affect CK activity, but GSH fully prevented the inhibitory effect of LGA ($F(5, 35) = 8.0$, $P < 0.001$) (Fig. 5).
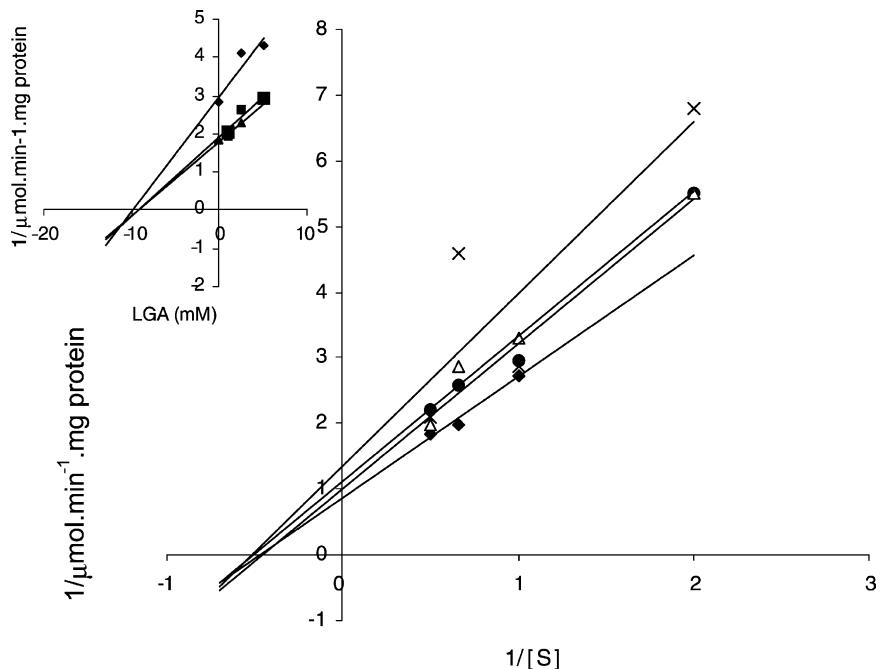
Fig. 3. Kinetic analysis of the inhibition of mitochondrial creatine kinase from cerebellum of rats by L-2-hydroxyglutaric acid. The graph shows a double reciprocal plot of creatine kinase for phosphocreatine concentrations (0.5–2.0 mM) [S] in the absence (◆ controls) and in the presence of 1.0 (●), 2.5 (△) and 5.0 (×) mM L-2-hydroxyglutaric acid. The inset shows the Dixon plot for Ki determination. All experiments were repeated at least three times and similar results were obtained. Data presented are representative of one experiment.
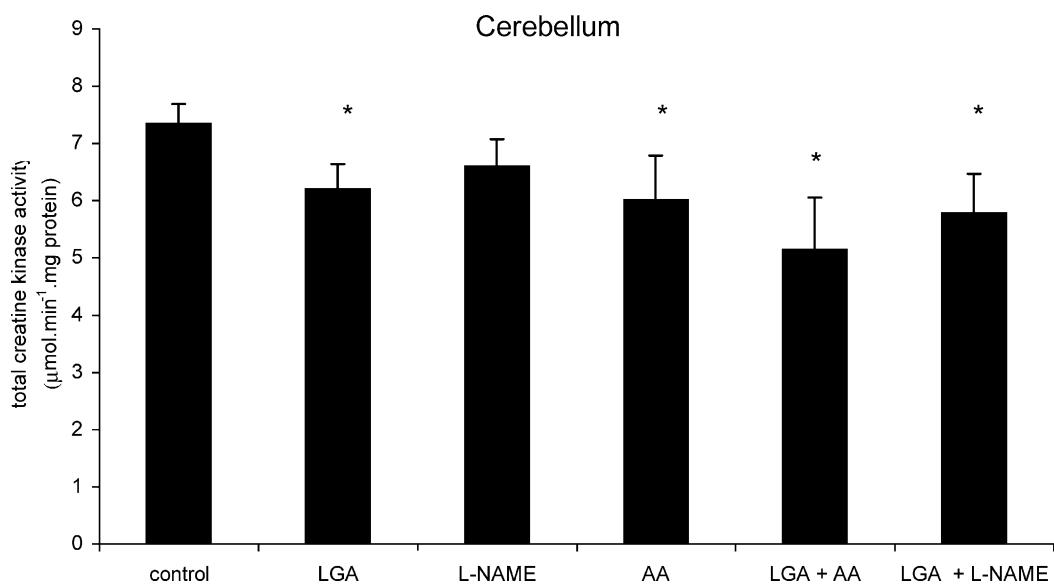
Fig. 4. Effect of ascorbic acid (AA) and L-NAME on total cerebellum CK activity in the presence or absence of L-2-hydroxyglutaric acid. Cerebellum homogenate preparations were pre-incubated at 37 °C for 15 min with 5.0 mM LGA, 1 mM AA, 5 mM LGA plus 1 mM AA, 1 mM L-NAME, or 5.0 mM LGA plus 1 mM L-NAME, and the enzyme activity was determined afterwards. Data are means ± S.D. for six independent experiments. $^*P < 0.05$ compared to control (one-way ANOVA followed by the Duncan's multiple range test).

4. Discussion

LGHA is a rare autosomal recessively inherited neurodegenerative disorder biochemically characterized by increased levels of LGA in urine, plasma and cerebrospinal fluid. Clinically, progressive ataxia combined with subcortical leukoencephalopathy and cerebellar atrophy on magnetic resonance imaging are observed in most patients (Barbot et al., 1997). However, the pathomechanisms evoking this destructive response, especially in the cerebellum,

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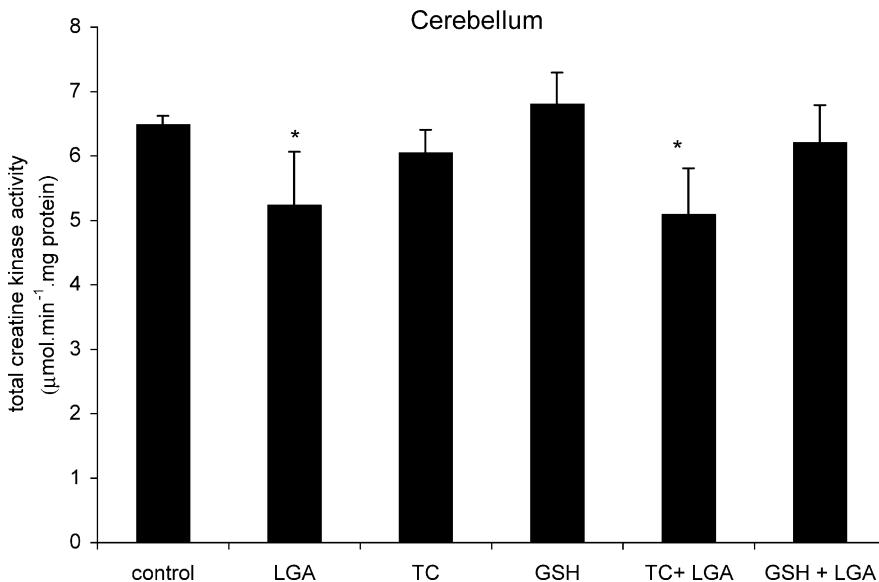
Fig. 5. Effect of α-tocopherol (TC) and reduced glutathione (GSH) on total creatine kinase activity from cerebellum in the presence or absence of L-2-hydroxyglutaric acid. Cerebellum homogenates were pre-incubated at 37 °C for 15 min with 5.0 mM LGA, 10 μM TC, 5.0 mM LGA plus 10 μM TC, 0.5 mM GSH, or 5.0 mM DGA plus 0.5 mM GSH, and the enzyme activity was determined afterwards. Data are means $\pm$ S.D. for eight independent experiments. $^{*}P < 0.05$ compared to control (one-way ANOVA followed by the Duncan's multiple range test).

are unknown and no specific therapy is available for this metabolic disease.

Although impairment of the phosphocreatine/CK system has been observed in neurodegenerative and mental diseases, such as Alzheimer's disease and schizophrenia (Burbaeva et al., 1999), very little is known about the influence of the metabolites accumulating in inherited neurodegenerative disorders on CK activities, which comprehend an important group of isoenzymes essential for the regulation of cellular energy homeostasis (Wallimann et al., 1992; Wyss et al., 1992).

In the present study, we investigated the in vitro influence of LGA on CK activities from cerebral cortex, cerebellum, skeletal muscle and cardiac muscle of young rats. We initially observed that LGA significantly inhibited tCK activity from cerebellum, but did not affect tCK from the other tissues studied. These results point to a selective action of LGA towards CK from cerebellum. This is a very interesting finding since patients with LHGA have severe and progressive degeneration of this cerebral structure. We cannot at the present explain why this important enzyme is inhibited by LGA only in the cerebellum, in contrast with the other cerebral structure (cortex) and tissues (cardiac and skeletal muscles) studied, where CK activity is also highly expressed. However, considering that spurious CK hybrids can be formed when brain tissue is prepared for CK activity measurement (Ramirez and Jiménez, 2000), a tentative explanation could be that distinct dimer associations may occur in the various tissues depending on the conditions of sample preparation and that the isoforms present in the cerebellum may be more susceptible to LGA effect.

Two of the five isoforms of CK are predominantly expressed in the brain: the cytoplasmic BB-CK and the mitochondrial Mia-CK (Molloy et al., 1992; Bessman and Geiger, 1981). We also demonstrated in the present study that LGA non-competitively inhibited Mi-CK activity from cerebellum in a dose–response manner, without suppressing the Cy-CK activity.

Mi-CK is part of a unique temporal and spatial energy buffer system in tissues with high energy requirements, being also important to inhibit the Ca^{+2}-induced opening of the mitochondrial permeability transition pore (PTP) which leads to apoptosis (O'Gorman et al., 1997; Kroemer et al., 1998; Crompton, 1999; Schlattner and Wallimann, 2000). Therefore, it can be presumed that the significant decline in Mi-CK activity provoked by LGA may induce a disturbance in energy metabolism in the cerebellum contributing to the cerebellum atrophy characteristic of LGHA.

It has been shown that CK activity decreases after brain exposure to agents promoting generation of free radicals probably by oxidation of the cysteine residues of the enzyme (Burmistrov et al., 1992; Wolosker et al., 1996; Arstall et al., 1998; Konorev et al., 1998; Stachowiak et al., 1998; Wallimann et al., 1998) and reagents reacting with thiols (Gross et al., 1996; Wolosker et al., 1996). Therefore, we investigated the effect of various antioxidants on the inhibitory effect of LGA on CK activity in order to test whether inhibition of CK activity by LGA was mediated by ROS. We verified that GSH, that acts as a naturally-occurring thiol-reducing agent (Meister and Anderson, 1983), fully prevented the inhibitory role of LGA on Mi-CK activity from cerebellum. This suggests that alterations in Mi-CK by LGA may be mediated by oxidation of sulfhydryl or other groups

of the enzyme. On the other hand, α-tocopherol, which is an excellent trapping agent for lipid peroxyl radicals (ROO^-) (Arstall et al., 1998), ascorbic acid, which traps hydroxyl and superoxide radicals (Konorev et al., 1998), and the nitric oxide synthase inhibitor L-NAME did not prevent LGA inhibitory activity, indicating that NO or other common free radicals are not involved in this inhibition.

It is interesting to note that ascorbic acid per se had a mild but significant inhibitory influence on the CK activity, a fact that was reported before in other systems and seems to be related to the ability of ascorbic acid, despite its known reducing action, to act as a pro-oxidant generating ROS in the presence of traces of transition metal ions in the reaction solutions and inducing lipid peroxidation and other free radical damage, including protein inactivation, when combined with iron (Halliwell and Gutteridge, 1990; Powers and Hamilton, 1999). In this context, it is well demonstrated that iron is found in high concentrations in the brain.

Cy-CK isoenzymes occur as dimers, in contrast with Mi-CK, which may appear as dimers or octamers (Molloy et al., 1992; Bessman and Geiger, 1981). The octameric Mi-CK isoform, localized in the intermembrane space of mitochondria (Wegmann et al., 1991), functionally interacts with the transmembrane proteins adenylate translocator (ANT) of the inner mitochondrial membrane and porin of the outer mitochondrial membrane (Wallimann et al., 1992; Wyss et al., 1992; Bessman and Carpenter, 1985). Various studies indicate that the octameric structure of Mi-CK is essential for its in vivo function and that dissociation of the octameric form impairs Mi-CK function necessary to sustain normal respiration (Schlattner and Wallimann, 2000; Khuchua et al., 1998). Furthermore, the SH- groups present on the enzyme have an important role in the association/dissociation of dimers and octamers, and the pH-dependence of the octamer dissociation suggests that SH-groups play an important role in the region of dimer contacts (Lipskaya, 2001). Taken together these observations and our present findings showing that LGA inhibits Mi-CK possibly through oxidation of critical thiol or other groups of the enzyme, it seems reasonable to postulate that LGA may induce destabilization of the octameric structure of Mi-CK with consequent loss of its functional capacity by oxidizing thiol groups necessary to maintain this structure.

In conclusion, we demonstrated in the present study that LGA significantly inhibits the mitochondrial creatine kinase activity from cerebellum in vitro probably though oxidation of essential groups of the enzyme. Although the brain concentrations of LGA in these patients are yet unknown and CSF and plasma LGA concentrations are of the order of 0.5 mM (Barth et al., 1993; Divry et al., 1993), we cannot exclude that even higher intracerebral concentrations of LGA may be attained in this neurometabolic disease, as possibly occurs in other similar organic acidemias so-called "cerebral" organic acidemias whose symptoms are predominantly or almost exclusively neurological (Hoffmann et al., 1994). Therefore, if the results presented in this report can be extrapolated to the human condition, it is conceivable that these concentrations may possibly affect energy utilization and/or production in the brain of these patients. However, further studies should be conducted to investigate whether in vivo administration of LGA provokes the same inhibition. If that is the case, the results of the present investigation may contribute to the understanding of the neuropathological findings of patients affected by LHGA.

References

Arstall, M.A., Bailey, C., Gross, W.L., Bak, M., Balligand, J.L., Kelly, R.A., 1998. Reversible *S*-nitrosation of creatine kinase by nitric oxide in adult rat ventricular myocytes. J. Mol. Cell. Cardiol. 30, 979–988.

Barbot, C., Fineza, I., Diogo, L., Maia, M., Melo, J., Guimarães, A., Pires, M.M., Cardoso, M.L., Vilarinho, L., 1997. L-2-Hydroxyglutaric aciduria: clinical. Brain Dev. 19, 268–273.

Barth, P.G., Hoffman, G.F., Jaeken, J., Lehnert, W., Hanefeld, F., van Gennip, A.H., Duran, M., Valk, J., Schutgens, R.B., Trefz, F.K., Reimann, G., Hartung, H.P., 1992. L-2-Hydroxyglutaric acidaemia: a novel inherited neurometabolic disorder. Ann. Neurol. 32, 66–71.

Barth, P.G., Hoffmann, G.F., Jaeken, J.J., Wanders, R.J., Duran, M., Jansen, G.A., Jakobs, C., Lehnert, W., Hanefeld, F., Valk, J., Schutgens, R.B.H., Trefz, F.K., Hartung, H.-P., Chamoles, N.A., Sfaello, Z., Caruso, U., 1993. L-2-Hydroxyglutaric acidemia: clinical and biochemical findings in 12 patients and preliminary report on L-2-hydroxyacid dehydrogenase. J. Inherit. Metab. Dis. 16, 753–761.

Barth, P.G., Wanders, R.J.A., Scholte, H.R., Abeling, N., Jakobs, C., Schutgens, R.B.H., Vreken, P., 1998. L-2-Hydroxyglutaric aciduria and lactic acidosis. J. Inherit. Metab. Dis. 21, 251–254.

Bessman, S.P., 1985. The creatine–creatine phophate energy shuttle. Annu. Rev. Biochem. 54, 831–862.

Bessman, S.P., Carpenter, C.L., 1985. The creatine–creatine phosphate energy shuttle. Annu. Rev. Biochem. 54, 831–865.

Bessman, S.P., Geiger, R.C., 1981. Transport of energy in muscle: the phosphorylcreatine shuttle. Science 211, 448–452.

Burbaeva, G.S., Savushkina, O.K., Dmitrievm, A.D., 1999. Brain isoforms of creatine kinase in health and mental diseases: Alzheimer's disease and schizophrenia. Vestn. Ross. Akad. Med. Nauk 1, 20–24.

Burmistrov, S.O., Mashek, O.P., Kotin, A.M., 1992. The action of acute alcoholic intoxication on antioxidant system and creatine kinase activity in the brain of rat embryos. Eksp. Klin. Farmakol. 55, 54–56.

Chen, E., Nyhan, W.L., Jakobs, C., Greco, C.M., Barkovich, A.J., Cox, V.A., Packman, S., 1996. L-2-Hydroxyglutaric aciduria: neuropathological correlations and first report of severe neurodegenerative disease and neonatal death. J. Inherit. Metab. Dis. 19, 335–343.

Cornish-Bowden, A., 1974. A simple graphical method for determining the inhibition constants of mixed. Biochem. J. 137, 143–144.

Crompton, M., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341, 233–249.

Divry, P., Jakobs, C., Vianey-Saban, C., Gibson, K.M., Michelakakis, H., Papadimitriou, A., Divari, R., Chabrol, B., Cournelle, M.A., Livet, M.O., 1993. L-2-Hydroxyglutaric aciduria: two further cases. J. Inherit. Metab. Dis. 16, 505–507.

Dixon, M., Webb, E.C., 1964. Enzymes, second ed. Longmans, London.

Duran, M., Kamerling, J.P., Barkker, H.D., van Gennip, H.A., Wadman, S.K., 1980. L-2-Hydroxyglutaric aciduria: an inborn error of metabolism? J. Inherit. Metab. Dis. 3, 11–15.

Fujitake, J., Ishikawa, Y., Fujii, H., Nishimura, K., Hayakawa, K., Inoue, F., Terada, N., Okochi, M., Tatsuoka, Y., 1999. L-2-Hydroxyglutaric aciduria: two Japanese adult cases in one family. J. Neurol. 246, 378–382.

Gross, W.L., Bak, M.I., Ingwall, J.S., Arstall, M.A., Smith, T.W., Balligand, J.L., Kelly, R., 1996. Nitric oxide inhibits creatine kinase

and regulates heart contractile reserve. Proc. Natl. Acad. Sci. U.S.A. 93, 5604–5609.

Halliwell, B., Gutteridge, J.M.C., 1990. Role of free radicals and catalytic metal ions in human disease. Methods Enzymol. 186, 1–85.

Hamman, B.L., Bittl, J.A., Jacobus, W.E., Aleen, P.D., Spencer, R.S., Tina, R., Ingwall, J.S., 1995. Inhibition of creatine kinase reaction decrease the contractile reserve of isolated rat hearts. Am. J. Physiol. 269, H1030–H1036.

Hemmer, W., Walliman, T., 1993. Functional aspects of creatine kinase in brain. Dev. Neurosci. 15, 249–260.

Hoffmann, G.F., Gibson, K.M., Trefz, F.K., Nyhan, W.L., Bremer, H.J., Rating, D., 1994. Neurological manifestations of organic acid disorders. Eur. J. Pediatr. 153 (Suppl. 1), S94–S100.

Hoffmann, G.F., Jakobs, C., Holmes, B., Mitchell, L., Becker, G., Hartung, H.P., Nyhan, W.L., 1995. Organic acids in cerebrospinal fluid and plasma of patients with L-2-hydroxyglutaric aciduria. J. Inherit. Metab. Dis. 18, 189–193.

Hughes, B.P., 1962. A method for estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin. Chim. Acta 7, 597–604.

Jacobus, W.E., 1985. Respiratory control and the integration of heart high-energy phosphate metabolism by mitochondrial creatine kinase. Annu. Rev. Physiol. 47, 707–725.

Khuchua, Z.A., Qin, W., Boero, J., Cheng, J., Payne, R.M., Saks, V.A., Strauss, A.W., 1998. Octamer formation and coupling of cardiac sarcomeric mitochondrial creatine kinase are mediated by charged N-terminal residues. J. Biol. Chem. 273, 22990–22996.

Konorev, E.A., Hogg, N., Kalyanaraman, B., 1998. Rapid and irreversible inhibition of creatine kinase by peroxynitrite. FEBS Lett. 427, 171–174.

Kroemer, G., Dallaporta, B., Resche-Rigon, M., 1998. Annu. Rev. Physiol. 60, 619–642.

Liao, R., Nascimben, L., Friedrich, J., Gawthmey, J.K., Ingwall, J.S., 1996. Decrease energy reserve in an animal model of dilated cardiomyopathy. Relationship to contractile performance. Circ. Res. 78, 893–902.

Lipskaya, T.Y., 2001. Mitochondrial creatine kinase: properties and function. Biochemistry (Moscow) 66, 1361–1376.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–267.

Meister, A., Anderson, M.E., 1983. Glutathione. Annu. Rev. Biochem. 52, 711–760.

Molloy, G.R., Wilson, C.D., Benfield, P., Vellis, J., Kumar, S., 1992. Rat brain creatine kinase messenger RNA levels are high in primary cultures of brain astrocytes and low in neurons. J. Neurochem. 9, 1925–1932.

O'Gorman, E., Beutner, G., Wallimann, T., Brdiczka, D., 1996. Differential effects of creatine depletion on the regulation of enzyme activities and on creatine-stimulated mitochondrial respiration in skeletal muscle. Biochim. Biophys. Acta 1276, 161–170.

O'Gorman, E., Beutner, G., Dolder, M., Korestsky, A.P., Brdicska, D., Wallimann, T., 1997. FEBS Lett. 414, 253–257.

Powers, S.K., Hamilton, K., 1999. Anti-oxidants and exercise. Clin. Sports Med. 18, 525–536.

Ramirez, O., Jiménez, E., 2000. Opposite transitions of chick brain catalytically active cytosolic creatine kinase isoenzymes during development. Int. J. Dev. Neurosci. 18, 815–823.

Schlattner, U., Wallimann, T., 2000. Octamers of mitochondrial creatine kinase isoenzymes differ in stability and membrane binding. J. Biol. Chem. 275, 17314–17320.

Schlegel, J., Zurbriggen, B., Wegamn, J., Wyss, N., Eppenberger, H.M., Walliman, T., 1988. Native mitochondrial creatine kinase forms octameric structures. I. Isolation of two interconvertible mitochondrial creatine kinase forms, dimeric and octameric mitochondrial creatine kinase: characterization, localization and structure-function relationships. J. Biol. Chem. 32, 16942–16953.

Stachowiak, O., Dolder, M., Wallimann, T., Richter, C., 1998. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J. Biol. Chem. 273, 16694–16699.

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., 1992. Intracellular compartmentation. Biochem. J. 281, 21–40.

Wallimann, T., Dolder, M., Schlattner, U., Eder, M., Hornemann, T., Kraft, T., Stolz, M., 1998. Creatine kinase: an enzyme with a central role in cellular energy metabolism. MAGMA 6, 116–119.

Wegmann, G., Huber, R., Zanolla, E., Eppenberger, H.M., Wallimann, T., 1991. Differential expression and localization of brain-type and mitochondrial creatine kinase isoenzymes during development of the chicken retina: Mi-CK as a marker for differentiation of photoreceptor cells. Differentiation 46, 77–87.

Wolosker, H., Panizzutti, R., Engelender, S., 1996. Inhibition of creatine kinase by S-nitrosoglutathione. FEBES Lett. 392, 274–276.

Wyss, M., Smeitink, J., Wevers, R.A., Wallimann, T., 1992. Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. Biochim. Biophys. Acta 1102, 119–166.

III. 6. Ácido D-2-hidroxiglutárico inibe a atividade da creatina quinase em músculo cardíaco e músculo esquelético de ratos jovens

D-2-Hydroxyglutaric acid inhibits creatine kinase activity from cardiac and skeletal muscle of young rats

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D-2-Hydroxyglutaric acid inhibits creatine kinase activity from cardiac and skeletal muscle of young rats

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Abstract

Background: Tissue accumulation of high amounts of D-2-hydroxyglutaric acid (DGA) is the biochemical hallmark of the inherited neurometabolic disorder D-2-hydroxyglutaric aciduria (DHGA). Besides severe neurological findings, patients affected by this disorder usually present hypotonia, muscular weakness, hypotrophy and cardiomyopathy. However, the underlying mechanisms of muscle injury in this disorder are virtually unknown.

Materials and methods: In the present study we have evaluated the *in vitro* role of DGA, at concentrations ranging from 0.25 – 5.0 mM, on total, cytosolic and mitochondrial creatine kinase activities from skeletal and cardiac muscle of 30-day-old Wistar rats.

Results: We verified that all creatine kinase activities were significantly inhibited by DGA (12-35% inhibition) at concentrations as low as 0.25 mM. We also observed that this inhibition was fully prevented by pre-incubation of the homogenates with reduced glutathione, suggesting that the inhibitory effect of DGA on CK activity is possibly mediated by modification of essential thiol groups of the enzyme.

Conclusion: Considering the importance of creatine kinase activity for cellular energy homeostasis, our results suggest that inhibition of this enzyme by increased levels of DGA might be an important mechanism involved in the myopathy and cardiomyopathy of patients affected by DHGA.

Key words: D-2-hydroxyglutaric acid, D-2-hydroxyglutaric aciduria, energy metabolism, creatine kinase, skeletal muscle, cardiac muscle.

Introduction

Creatine Kinases (CKs, EC 2.7.3.2) comprehend a family of enzymes catalysing the reversible transfer of a phosphoryl group between ATP and creatine [1]. CKs are present in high quantities in tissues that have high-energy flux or requirements such as skeletal muscle, cardiac muscle and brain [2]. They are formed by a group of isoenzymes consisting of the cytosolic dimers BB-CK (brain), MM-CK (muscle) and the heterodimer MB-CK, which is present in cardiac muscle cytosol. In addition, there are two mitochondrial CKs (Mi-CK) positioned in the intermembrane space, termed Mib-CK (specific for sarcomeric muscle) and Mia-CK (the ubiquitous isoform) [3]. A functional interplay between mitochondrial and cytosolic isoforms of CK is thought to be important for the regulation of cellular energy homeostasis. The cytosolic isoenzymes re-phosphorylate the locally produced free ADP from phosphocreatine, releasing ATP and creatine. The mitochondrial CKs catalyse the reaction of creatine to phosphocreatine at the expense of mitochondrial ATP [1,3-5]. In most tissues containing CK activity, the cytosolic and mitochondrial CK isoenzymes are coexpressed. [3]. Because of the central role of the phosphocreatine/CK system in the

regulation of cellular ATP levels, alterations in CK activity have been proposed to explain the pathophysiology of some neurodegenerative disorders presenting altered energy metabolism [6]. Furthermore, impaired CK function seems to be of significance for the development of myocardial diseases [7-9].

D-2-hydroxyglutaric aciduria (DHGA) is a rare organic aciduria first described in 1980 [10]. The disorder has now been identified in at least 26 patients [11-13]. The clinical phenotype in DHGA varies from neonatal intractable epilepsy to mild developmental delay. Two variants have been recognized [10-11]. Patients affected by the severe variant suffer from a severe encephalopathy with developmental delay, epilepsy and hypotonia. Dystonic or choreoathetotic movement disorders and cardiomyopathy with cardiomegaly, hypertrophy and reduced contractility have been also observed in many patients with this variant [14-16]. One-third of these patients die during infancy [10,11]. The other variant of DHGA is characterized by a mild phenotype, which essentially shares the same characteristics of the severe phenotype [10].

Biochemically, this disorder is characterized by increased tissue accumulation and urinary excretion of D-2-hydroxyglutaric acid (DGA) [10,11]. However, the biochemical
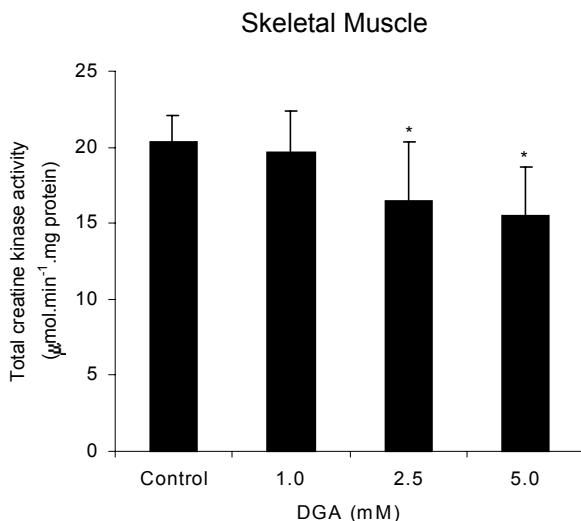
Skeletal Muscle

Fig. 1. Effect of D-2-hydroxyglutaric acid (DGA) on total creatine kinase (tCK) activity in skeletal muscle homogenates of 30-day-old rats. Values are means ± SD for n = 6 independent experiments (animals)per group of creatine kinase activity measured in the presence or absence of the metabolite. * p< 0.05 compared to control (one-way ANOVA followed by the Duncan´s multiple range test).

defect involved in DHGA is so far unknown. Moreover, virtually nothing is known about the pathophysiology of the tissue damage found in patients with DHGA. However, considering that elevated levels of lactate and Krebs cycle intermediates also accumulate in plasma, CSF and urine of patients affected by this disorder, it is conceivable that energy metabolism dysfunction may be associated with the disease [10,11,16].

Previous results from our laboratory have shown that DGA significantly inhibits CK activities in cerebral cortex of young rats [17]. Therefore, considering that CK plays an important role in cellular energy homeostasis and that impaired CK function is of significance for the development of different pathological states [7-9], in the present study we investigated the *in vitro* effects of DGA on CK activities in total homogenates (tCK) from skeletal and cardiac muscle of young rats, as well as in the cytosolic (Cy-CK) and mitochondrial (Mi-CK) fractions prepared from these tissues, in the hope to clarify the pathogenetic mechanisms of muscular injury in DHGA. We also examined the influence of the antioxidant glutathione, the free radical scavenger α-tocopherol, and the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) on the effects produced by DGA on CK activity since the enzyme contains thiol groups which are critical to its function and that can be oxidized and are susceptible to oxidative stress.

Material and methods

Subjects and Reagents

Thirty-day-old Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, were housed in groups of eight with their mothers on the day of birth. They were maintained on a 12:12 h light/dark cycle (lights on 07.00-19:00 h) in an air-conditioned constant temperature (22°C $\pm$ 1°C) colony room, with free access

to water and a 20 % (w/w) protein commercial chow (Germani, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre and followed the "Principles of Laboratory Animal Care (NIH publication 85-23, revised 1985).

All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA. D-2-hydroxyglutaric acid (DGA) and the other acids tested were dissolved in Tris-HCL buffer, pH 7.5, for the enzymatic technique on the day of the experiments.

Preparation of total homogenates and the mitochondrial and cytosolic fractions

For the preparation of total homogenates, rats were sacrificed by decapitation, the skeletal and cardiac muscles were isolated and homogenized in 10 volumes of saline solution, pH 7.5, using a ground glass type Potter-Elvejhem homogenizer immersed in an iced box. At least two homogenizations of 30-s duration were performed at approximately 1000 ms/min with an electrically driven Teflon pestle. The homogenates were centrifuged at 800 x g for 10 min at 4°C and the pellet discarded [18]. A part of the supernatants was used for the estimation of total CK (tCK) activity. For the preparation of mitochondrial and cytosolic fractions, the supernatants were centrifuged at 27 000 x g for 30 min at 4°C in a Sorval DC-2B centrifuge. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the mitochondrial creatine kinase (Mi-CK) enzymatic assay. The supernatants were further centrifuged at 125,000 x g for 60 min at 4°C in a OTD-65B Sorval centrifuge, the microsomal pellet discarded, and the cytosol (supernantant) was used for the cytosolic creatine kinase (Cy-CK) enzymatic assay. The cytosolic fraction did not contain contaminating Mi-CK, as observed by immunoblotting. Samples were stored at – 40° C and the maximal period between total homogenate and sub-cellular preparations and enzyme analysis was always less than 5 days. . We have previously observed that under these conditions CK activity is not altered.

Cardiac Muscle
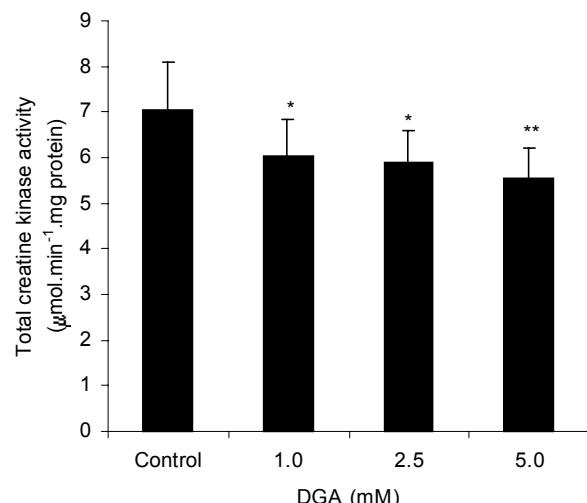
Fig. 2. Effect of D-2-hydroxyglutaric acid (DGA) on total (tCK) creatine kinase activity in cardiac muscle homogenates of 30-day-old rats. Values are means ± SD for n = 8 independent experiments (animals) per group of creatine kinase activity measured in the presence or absence of the metabolite. * p< 0.05, ** p< 0.01 compared to control (one-way ANOVA followed by the Duncan´s multiple range test).

Skeletal Muscle

Cardiac Muscle

homogenates were pre-incubated with 0.5 mM GSH, 1 mM L-NAME or 10 μM trolox final concentrations for 15 min alone or in the presence of DGA, after which the other reagents were supplemented and reaction carried out. The concentrations of the antioxidants used in these assays were similar to those previously described [20-22]. None of the substances supplemented to the assay medium interfered with the color development or spectrophotometer reading. Results were expressed as μmol of creatine formed per min per mg protein.

Protein determination

Protein was measured by the method of Lowry *et al.* [23] using bovine serum albumin as standard.
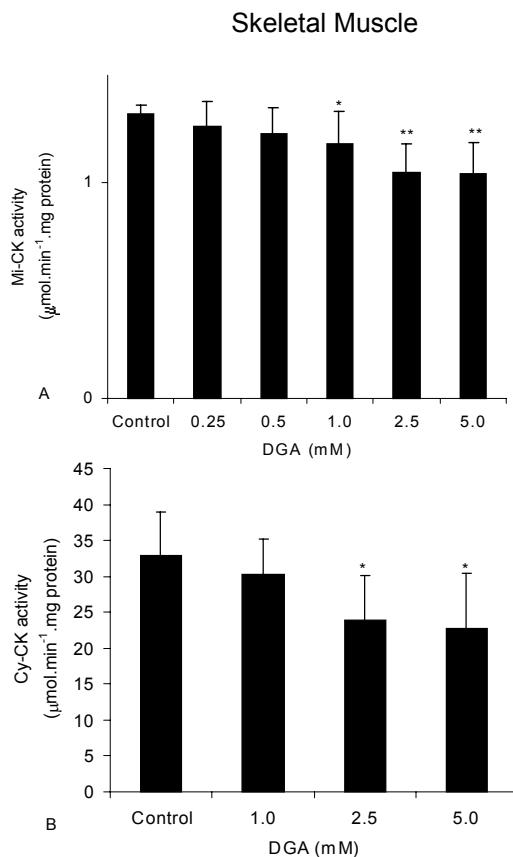
Fig. 3. Effect of D-2-hydroxyglutaric acid (DGA) on creatine kinase activities in mitochondrial (Mi-CK) (A) and cytosolic (Cy-CK) (B) preparations from skeletal muscle preparations of 30-day-old rats. Values are means ± SD for n = 6 independent experiments (animals) per group of creatine kinase activity measured in the presence or absence of the metabolite. * p< 0.05, **p<0.01 compared to control (one-way ANOVA followed by the Duncan´s multiple range test).

Creatine Kinase Activities

CK activity was measured in total homogenates, as well as in the cytosolic and mitochondrial preparations from cardiac and skeletal muscle. The reaction mixture consisted of the following medium: 60 mM Tris-HCl buffer, pH 7.5, containing 7 mM phosphocreatine, 9 mM MgSO$_4$, and approximately 0.05-1.0 μg protein in a final volume of 0.1 mL. For enzymatic analysis in mitochondrial fractions, 0.625 mM lauril maltoside was added to the incubation mixture. DGA, at 0.25 to 5 mM final concentrations, was also supplemented to the medium, whereas controls did not contain the acid. After 15 minutes of pre-incubation at 37°C, the reaction was started by the addition of 0.3 μmol ADP plus 0.08 μmol reduced glutathione. The reaction was stopped after 10 minutes by the addition of 1 μmol   p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls containing all components of the incubation medium except homogenates were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes [19]. The color was developed by the addition of 0.1 mL 2 % α-naphtol and 0.1 mL 0.05 % diacetyl in a final volume of 1 mL and read after 20 minutes at 540 nm. In some experiments,

Fig. 4. Effect of D-2-hydroxyglutaric acid (DGA) on creatine kinase activities in mitochondrial (Mi-CK) (A) and cytosolic (Cy-CK) (B) preparations from cardiac muscle of 30-day-old rats. Values are means ± SD for n = 7 independent experiments (animals) per group of creatine kinase activity measured in the presence or absence of the metabolite. * p< 0.05, **p<0.01 compared to control (one-way ANOVA followed by the Duncan's multiple range test).

Fig. 5- Effect of reduced glutathione (GSH), α-tocopherol (TC) and Nω-nitro-L-arginine methyl ester (L-NAME) on total creatine kinase (tCK) activity from skeletal muscle of 30-day-old rats in the presence or absence of D-2-hydroxyglutaric acid (DGA). Skeletal muscle homogenates were pre-incubated at 37°C for 15 min with 5.0 mM DGA, 0.5 mM GSH, 5.0 mM DGA plus 0.5 mM GSH, 10 μM TC, 5.0 mM DGA plus 10 μM TC, 1.0mM L-NAME, 5.0 mM DGA plus 1.0 mM L-NAME, and the enzyme activity was determined afterwards. Data are means ± SD for 4 independent experiments (animals). **$p < 0.01$ compared to control (one-way ANOVA followed by the Duncan's multiple range test).

Statistical analysis

Unless otherwise stated, results are presented as means ± standard deviation. All assays were performed in triplicate and the median was used for statistical analysis. Data from the activity of creatine kinase were analyzed using the one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. Differences between the 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.

Results

We first examined the effect of DGA, at concentrations varying from 1 to 5 mM, on CK activity in total homogenates (tCK) from skeletal and cardiac muscle. Values for these activities in controls were similar to those described previously [24]. DGA significantly inhibited the enzyme activity from skeletal muscle (up to 24 %) [$F_{(3,23)}=3.811;p<0.05$] at concentrations of 2.5 mM and higher (Figure 1) and cardiac muscle (up to 22 %) [$F_{(3,31)}=4.999;p<0.01$] at all DGA concentrations used (Figure 2). It can be also seen that tCK activity was greater in skeletal muscle than in cardiac muscle.

We then investigated the effect of DGA, at concentrations varying from 0.25 to 5 mM, on CK activity from the mitochondrial (Mi-CK) and cytosolic (Cy-CK) fractions of rat skeletal muscle. It can be seen that DGA significantly inhibited both Mi-CK activity (up to 24%) at concentrations of 1.0 mM and greater [$F_{(5,23)}=3.05; p<0.01$] (Figure 3A) and Cy-CK

activity (up to 30%) at concentrations of 2.5 mM and higher [$F_{(3,23)}=3.603; p<0.05$] (Figure 3B).

Next, we investigated the effect of DGA at concentrations varying from 0.25 to 5 mM on CK activity from the mitochondrial (Mi-CK) and cytosolic (Cy-CK) fractions of rat cardiac muscle. It can be seen that the metabolite significantly inhibited Mi-CK activity (up to 35%) [$F_{(5,41)}=4.312; p<0.01$] (Figure 4A), and Cy-CK activity (up to 28%) at concentrations as low as 0.25 mM [$F_{(5,41)}=5.136, p<0.01$] (Figure 4B). These results indicate that cardiac muscle CK activities are more vulnerable to DGA inhibitory action. It can be also seen in Figures 3 and 4 that the cytosolic CK activities were various orders of magnitude higher than the mitochondrial CK activities, and this is in agreement with the literature [25,26].

In order to test whether the significant reduction of CK activity caused by DGA was due to a non-specific effect of D-enantiomers or acidic compounds, we then investigated the effect of the same concentrations of α-ketoglutaric acid (KGA) and D-hydroxymethyllvaleric, organic acids with similar molecular mass and chemical structure to DGA, on tCK activity in homogenates. We observed that both acids caused no alteration on this activity in homogenates from skeletal muscle [$F_{(4,19)}=0.392$ $p>0.05$] and cardiac muscle [$F_{(4,17)}=0.209$ $p>0.05$].

We further evaluated whether the DGA inhibitory activity was mediated by oxidation of critical (–SH) groups on the enzyme, by preincubating cardiac and skeletal muscles homogenates in the presence of the antioxidant glutathione (GSH), the free radical scavenger α-tocopherol and the nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). The enzyme activity was measured in the whole cell

homogenates since free radicals can be better produced with the whole cell machinery. First, by-preincubating skeletal muscle homogenate in the presence of the antioxidants glutathione (0.5 mM GSH), α-tocopherol (10 μM), or the NOS inhibitor L-NAME (1 mM), alone or combined with 5 mM DGA. We verified that these drugs *per se* did not affect tCK activity in this tissue, in contrast to DGA, which significantly inhibited the enzyme activity $[F(7,31)=5.939, p<0.0001]$ (Figure5). Furthermore, GSH was able to prevent the inhibitory effect of DGA, in contrast to the other antioxidants. We also tested the influence of these antioxidants on the DGA-induced inhibitory activity in cardiac muscle homogenates and similar results were obtained $[F(7,23) = 9.087, p<0.0001]$ (Figure 6). These results indicate that reduction of sulfhydryl groups of the enzyme by antioxidants protect tCK activity, and that nitric oxide or other common free radicals scavenged by α-tocopherol were not involved in this inhibition.

Discussion

DGA accumulates in urine, plasma and cerebrospinal fluid of individuals affected by DHGA, an inherited metabolic disorder clinically characterized by neurological dysfunction, myopathy and cardiomyopathy [10-13]. The pathomechanisms underlying this symptomatology are poorly known and no specific therapy is available for this metabolic disease [10,11,16]. However some data in the literature indicate that mitochondrial dysfunction might be involved in the pathogenetic mechanisms of this disease. In this context, it has been observed that patients with

DHGA excrete large quantities of Krebs cycle intermediates [10,11,13], and that DGA decreases glucose utilization, CO_2 formation and the activities of complexes IV and V of the respiratory chain in the brain [16,27]. We have recently demonstrated that DGA significantly inhibits CK activity from rat cerebral cortex, strengthening the view that energy metabolism is disrupted by this metabolite [17]. Based on the central role of the phosphocreatine/CK system in the regulation of energy metabolism and considering that Mi-CK is functionality coupled to the mitochondrial adenine nucleotide translocator (ANT) and to oxidative phosphorylation (OXPHOS), we postulated that brain dysfunction of DHGA patients could be at least in part due to energy deficit.

In the present study we extended our previous investigations by evaluating the *in vitro* effects of DGA on the mitochondrial and cytosolic CK activities from skeletal and cardiac muscle because hypotonia, neuromuscular weakness and cardiomyopathy are commonly observed in DHGA and reduction of CK activity may lead to selective tissue damage.

We firstly observed that the activities of tCK, Mi-CK and Cy-CK and their respective proportions were the same as those previously described. Total CK activity in cardiac muscle was about 25% of that in skeletal muscle. Furthermore, Cy-CK and Mi-CK comprehend respectively $\cong 70\%$ and 25 % of total CK activity in heart, as opposed to 95% and 5% in skeletal muscle [25,26].

We demonstrated that all CK activities (tCK, Mi-CK and Cy-CK) from cardiac and skeletal muscle were significantly inhibited by DGA at concentrations similar to those found in the
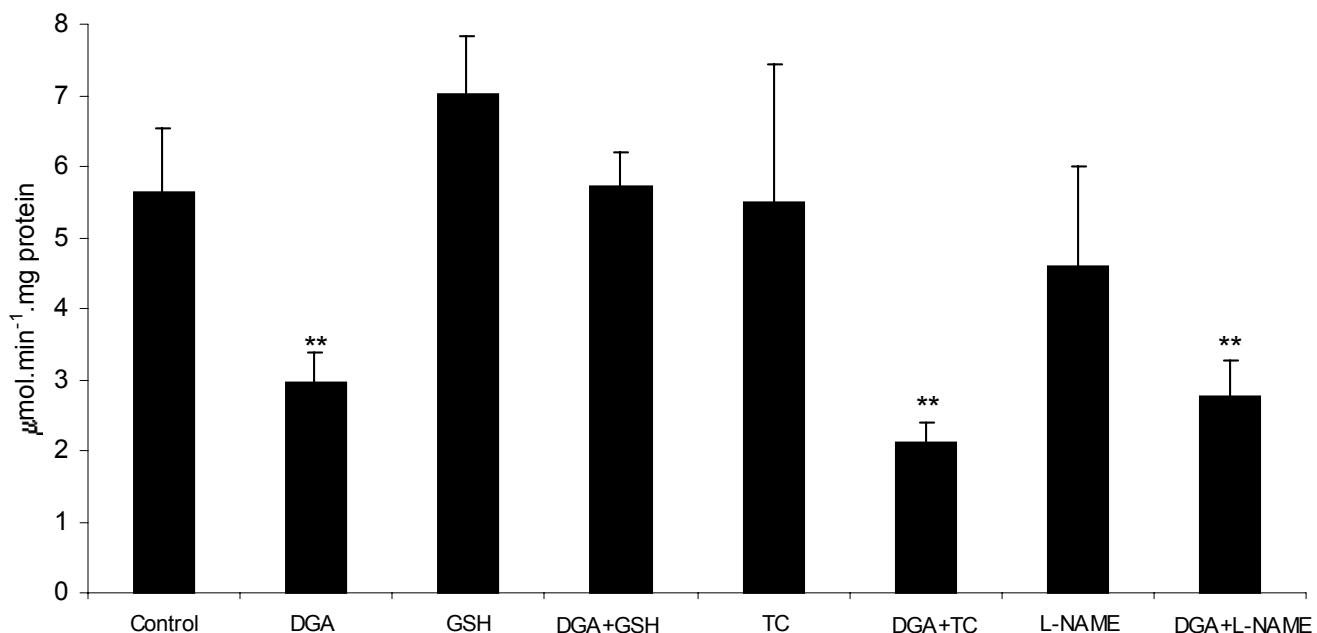
Fig. 6- Effect of reduced glutathione (GSH), α-tocopherol (TC) and Nω-nitro-L-arginine methyl ester (L-NAME) on total creatine kinase (tCK) activity from cardiac muscle of 30-day-old rats in the presence or absence of D-2-hydroxyglutaric acid (DGA). Cardiac muscle homogenates were pre-incubated at 37°C for 15 min with 5.0 mM DGA, 0.5 mM GSH, 5.0 mM DGA plus 0.5 mM GSH, 10 μM TC, 5.0 mM DGA plus 10 μM TC, 1.0 mM L-NAME, 5.0 mM DGA plus 1.0 mM L-NAME, and the enzyme activity was determined afterwards. Data are means ± SD for 3 independent experiments (animals). **p < 0.01 compared to control (one-way ANOVA followed by the Duncan's multiple range test).

blood and CSF (up to 757 μmol/L) of patients affected by DHGA [10,11]. It is interesting to note that cardiac muscle CK activities were apparently more affected by DGA since the inhibition occurred with doses as low as 0.25 mM DGA, whereas in the skeletal muscle only occurred with 1 mM and higher doses of DGA. It is tempting to speculate that this might possibly explain why cardiomyopathy affects a great deal of patients with the severe variant of the disease.

We also found that α-ketoglutaric acid (KGA) and D-hydroxymethylvaleric, organic acids with similar molecular mass and chemical structure to DGA, did not affect tCK activity from cardiac and skeletal muscle, a fact that indicates a specificity for DGA action.

Our previous findings showed that mitochondrial and cytosolic CK activities from cerebral cortex were inhibited by DGA [17], and this is in agreement with the present results where both activities were inhibited in skeletal and cardiac muscle.

As regards to the consequences of our present findings, it should be emphasized that CK function is crucial to maintain a high concentration of ATP and low concentrations of the products of ATP hydrolysis, ADP, Pi and H$^+$, in cells, ensuring that |ΔG$_{ATP}$| will be adequate to maintain ion gradients and perform the cellular mechanical work [28]. These observations may explain the ability of excitable tissues, such as heart and skeletal muscle to cope with changing energy consumption rates during periods of increased performance [29]. Furthermore, depression of CK activity in the heart has been implicated in the pathogenesis of cardiomyopathy and heart failure [30,31], compromising contractile function and end-diastolic pressure at high workloads [32-34]. It has been also demonstrated that the net result of CK activity reduction is a decreased capacity of the heart to synthesize ATP from PCr as much as 70% [35-36].

Furthermore, inhibition of CK in skeletal muscle by creatine analogues [37-40] or in CK "knockout" mouse models with loss of both cytosolic MCK and Mi-CK [41] have been shown to lead to significant contractile abnormalities, as well as alterations in muscle cell size, tubular aggregates of sarcoplasmic reticulum membranes, mitochondrial volume and size and also of glycolytic capacity. In addition, skeletal muscle of CK "knockout" models are unable to hydrolyze PCr [41], whereas the cardiac muscle from these animals are incapable of maintaining [ADP] and |ΔG$_{ATP}$| at baseline levels [42]. These studies clearly demonstrate the important role of CK in normal muscle function. Therefore, it can be presumed that the significant decline in all CK activities provoked by DGA, as demonstrated in the present study, may induce a disturbance in energy metabolism contributing to the myopathy and cardiomyopathy characteristic of DGHA.

It has been demonstrated that CK is sensitive to oxidative modification [43] and that its activity decreases after exposing brain to agents promoting free radical generation [44]. NO, peroxynitrite and other free radicals can also inactivate the enzyme in skeletal muscle [45] and also in cardiac muscle [46-49]. Therefore, we tested the influence of various antioxidants on the inhibitory effect of DGA on CK. We verified that GSH, that acts as a naturally-occurring thiol-reducing agent [50], fully prevented the inhibitory role of DGA on skeletal and cardiac muscle CK activity. Therefore, it is likely that this inhibition may be mediated by oxidation of sulfhydryl or other groups of the enzyme. These results are in accord with other studies showing that the free radical-induced inhibition of cardiac mitochondrial CK can be reversed *in vitro* by the reducing agents dithiothreitol [8,46] and 2-mercaptoethanol [47]. On the other hand, it could be also presumed that DGA, by inhibiting the Mi-CK activity probably by oxidation of thiol groups, may possibly disrupt the

octameric CK isoform, which is considered to be the most active and important for its *in vivo* function [51]. In this scenario, it is thought that only the octameric Mi-CK is able to interact simultaneously with two opposing membranes, forming complexes with ANT and porin in the so-called mitochondrial contact sites between the outer and the inner mitochondrial membranes. These contact sites play an important role for efficient energy export into the cytosol by direct channeling of substrates and products in between the interaction partners [52]. Destabilization of the Mi-CK octamer leads to an impairment of Mi-CK function [53].

Our present findings are therefore indicative that DGA possibly acts though modification of critical thiol or other groups present in the enzyme molecule and necessary for its function. Furthermore, it can be also concluded that, under our *in vitro* assay conditions, NO or other common free radicals are not involved in the inhibitory effect of DGA in muscle CK activity, since the NO synthase inhibitor L-NAME and the free radical scavenger α-tocopherol did not prevent DGA inhibitory effect. These results are similar to our previous experiments performed in cerebral cortex from young rats [17].

Although the precise mechanisms by which DGA suppresses Mi-CK activity cannot be at present fully established, oxidation or other alteration of important groups of the enzyme is a possibility. In this context, CK contains eight sulfhydryl groups, and several reagents acting on these groups are able to inactivate the enzyme [54]. There is a highly conserved cysteine reside located near the catalytic site of CK, which is crucial for full enzyme activity [55]. It probably interacts with the guanidine group of the creatine substrate and is most likely responsible for its correct orientation within the active site [56]. This cysteine residue was identified as the main target for chemical inactivation of CK by a large number of reagents [57,58].

However, further studies will be required to investigate whether a direct effect of DGA on the enzyme CK occurs and also whether the *in vitro* inhibition here observed can be also demonstrated *in vivo*.

In conclusion, we demonstrated here that DGA significantly inhibits both mitochondrial and cytosolic creatine kinase activities from cardiac and skeletal muscles *in vitro*. Considering that this enzyme is crucial for buffering ATP levels and transport between the sites of ATP generation and consumption, especially in tissues enriched in mitochondria and with a high energy demand, the results of the present investigation may possibly contribute to clarify the pathophysiology of the myopathy, characterized by progressive muscle weakness and by electromyography alterations, and the cardiomyopathy, occurring in the severe form of DHGA, especially during crises where the levels of DGA increase dramatically. Finally, it will be important to perform further studies to evaluate whether compounds that either buffer intracellular energy stores (creatine) or increase overall mitochondrial function (coenzyme Q$_{10}$) and antioxidants may benefit patients with DHGA.

References

1   Bessman SP. The creatine-creatine phosphate energy Shuttle. Annu Rev Biochem 1985; 54:831-862.
2   Schlegel J, Zurbriggen B, Wegamn J, Wyss N, Eppenberger HM, Walliman T. Native mitochondrial creatine kinase forms octameric structures. I. Isolation of two interconvertible mitochondrial creatine kinase forms, dimeric and octameric mitochondrial creatine kinase: Characterization, localization and structure-function relationships. J Biol Chem 1988; 32:16942-16953.
3   Wallimann T, Wyss M, Brdiczka D, Nicolay K. Intracellular compartmentation, structure and function of creatine kinase

isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem J 1992; 281:21-40.

4   Wyss M, Smeitink J, Wevers R A, Wallimann T. Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. Biochim Biophys Acta 1992; 1102:119-166.

5   Jacobus WE. Respiratory control and the integration of heart high-energy phosphate metabolism by mitochondrial creatine kinase. Annu Rev Physiol 1985; 47: 707-725.

6   Hemmer W and Walliman T. Functional aspects of creatine kinase in brain. Dev Neurosci 1993; 15:249-260.

7   Hamman BL, Bittl JA, Jacobus WE, Aleen PD, Spencer, RS, Tina R, et al. Inhibition of creatine kinase reaction decrease the contractile reserve of isolated rat hearts. Am J Physiol. 1995; 269:H1030-H1036.

8   Gross WL, Bak MI, Ingwall JS, Arstall MA, Smith TW, Balligand JL, et al. Nitric oxide inhibits creatine kinase and regulates heart contractile reserve. Proc Natl Acad Sci. USA 1996; 93:5604-5609.

9   Liao R, Nascimben L, Friedrich J, Gawthmey JK, Ingwall JS. Decrease energy reserve in an animal model of dilated cardiomyopathy. Relationship to contractile performance. Circ Res 1996; 78:893-902.

10 van der Knaap MS, Jakobs C, Hoffmann GF, Nyhan WL, Renier WO, Smeitink JAM, et al. D-2-Hydroxyglutaric aciduria: biochemical marker or clinical disease entity? Ann Neurol 1999; 45:111-119.

11 van der Knaap MS, Jakobs C, Hoffman GF, Duran M, Muntau AC, Schweitzer S, Kelley RI, Parrot-Rouland F, Amiel J, De Lonlay P, Rabier D, Eeg-Olofsson O. D-2-Hydroxyglutaric aciduria: further clinical delineation. J Inherit Metab Dis 1999; 22:404-413.

12 Chalmers RA, Lawson AM, Watts RWE, Tavill AS, Kamerling JP, Hey E, et al. D-2-Hydroxyglutaric aciduria: case report and biochemical studies. J Inherit Metab Dis 1980; 3:11-15.

13 Wajner M, Vargas CR, Funayama C, Fernandez A, Elias ML, Goodman SI, et al. D-2-hydroxyglutaric aciduria in a patient with a severe clinical phenotype and unusual MRI findings. J Inherit Metab Dis 2002; 25:28-34.

14 Eeg-Olofsson O. D-2-hydroxyglutaric aciduria with cerebral, vascular, and muscular abnormalities in a 14-year-old boy. J Child Neurol 2000; 15:488-492.

15 Sugita K, Kakinuma H, Okajima Y, Ogawa A, Watanabe H, Niimi H. Clinical and MRI findings in a case report of D-2-hydroxyglutaric aciduria. Brain Dev 1995; 17:139-141.

16 Kölker S, Pawlak V, Ahlemeyer B, Okun JG, Horster F, Mayatepek E, et al. NMDA receptor activation and respiratory chain complex V inhibition contribute to neurodegeneration in D-2-hydroxyglutaric aciduria. Eur J Neurosci 2002; 16:21-28.

17 Silva CG, Bueno ARF, Schuck PF, Leipnitz G, Ribeiro CJA, Rosa RB, et al. Inhibition of creatine kinase activity from rat cerebral cortex by d-2-hydroxyglutaric acid in vitro. Neurochem. Int. *in press*

18 Ramirez O and Jiménez E. Opposite transitions of chick brain catalytically active cytosolic creatine kinase isoenzymes during development. Int J Dev Neurosci 2000; 18:815–823.

19 Hughes BP. A method for estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin Chim Acta 1962; 7:597-604.

20 Stanimirovic DB, Wong J, Ball R, Durkin JP. Free radical-induced endothelial membrane dysfunction at the site of blood-brain barrier: relationship between lipid peroxidation, Na,K-ATPase activity, and 51Cr release. Neurochem Res 1995; 20:1417-1427.

21 Avrova NF, Victorov IV, Tyurin VA, Zakharova IO, Sokolova TV, Andreeva NA, et al. Inhibition of glutamate-induced intensification of free radical reactions by gangliosides: possible role in their protective effect in rat cerebellar granule cells and brain synaptosomes. Neurochem Res 1998; 23:945-952.

22 Kölker S, Okun JG, Ahlemeyer B, Wyse ATS, Hörster F, Wajner M, 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.

23 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-267.

24 O'Gorman E, Beutner G, Wallimann T, Brdiczka D. Differential effects of creatine depletion on the regulation of enzyme activities and on creatine-stimulated mitochondrial respiration in skeletal muscle. Biochim Biophys Acta.1996; 1276:161-170.

25 Veksler V, Kuznetsov V, Anflous K, Mateo P, van Deurson J, Wieringa B, et al. Muscle creatine kinase deficient mice. J Biol Chem 1995; 270:19921–19929.

26 Bittl J, DeLayre J, Ingwall J. Rate equation for creatine kinase predicts the in vivo reaction velocity: 31P NMR surface coil studies in brain, heart, and skeletal muscle of the living rat. Biochemistry 1987; 26:6083– 6090.

27 Silva CG, Ribeiro CAJ, Leipnitz G, Dutra-Filho CS, Wyse ATS, Wannmacher CMD, et al. Inhibition of cytochrome *c* oxidase activity in rat cerebral cortex and human skeletal muscle by D-2-hydroxyglutaric acid in vitro. Biochim Biophys Acta 2002: 1586:81-91.

28 Kammermeier H. High energy phosphate of the myocardium: concentration versus free energy change. Basic Res Cardiol 1987;82(suppl 2): 31–36.

29 Saks VA, Ventura-Clapier R, Aliev MK. Metabolic control and metabolic capacity: two aspects of creatine kinase functioning in the cells. Biochim Biophys Acta 1996; 1274:81-88.

30 Veksler V and Ventura-Clapier R. In situ study of myofibrils, mitochondria and bound creatine kinases in experimental cardiomyopathies. Mol Cell Biochem 1994; 133/134:287-298.

31 Saks VA, Belikova YO, Kuznetsov AV, Khuchua ZA, Branishie TH, Semenovsky ML, et al. Phosphocreatine pathway for energy transport: ADP diffusion and cardiomyopathy. Am J Physiol 1991; 261(suppl.):30-38.

32 Fossel ET and Hoefeler H. Complete inhibition of creatine kinase in isolated perfused rat hearts. Am J Physiol 1987; 252:E124-E130.

33 Wallimann T, Dolder M, Schlattner U, Eder M, Hornemann T, Kraft T, et al. Creatine kinase: An enzyme with a central role in cellular energy metabolism. MAGMA 1998; 6:11-119.

34 Tian R and Ingwall JS. Energetic basis for reduced contractile reserve in isolated rat hearts. Am J Physiol 1996; 270:H1207-H1216.

35 Liao R, Nascimben J, Friedrich J, Gwathmey JK, Ingwall JS. Decreased energy reserve in an animal model of dilated cardiomyopathy: relationship to contractile performance. Circ Res 1996; 78:893–902.

36 Nascimben L, Ingwall J, Pauletto P, Friedrich J, Gwathmey J, Saks Pessina A, et al. Creatine kinase system in failing and nonfailing human myocardium. Circulation 1996; 94:1894 –1901.

37 Fitch CD, Jellinek M, Gitts RH, Baldwin KM, Holloszy JO. Phosphorylated beta-guanidinopropionate as a substitute for phosphocreatine in rat muscle. Am J Physiol 1975; 228:1123–1125.

38 Shoubridge EA and Radda GK. A 31P-nuclear magnetic resonance study of skeletal muscle metabolism in rats depleted of creatine with the analogue beta-guanidinopropionic acid. Biochim Biophys Acta 1984; 805:79–88.

39 Meyer RA, Brown TR, Krilowicz BL, Kushmerick MJ. Phosphagen and intracellular pH changes during contraction of creatine-depleted rat muscle. Am J Physiol 1986; 250:C264–C274.

40 van Deursen J, Heerschap A, Oerlemans F, Ruitenbeek W, Jap P, ter Laak H, et al. Skeletal muscles of mice deficient in muscle creatine kinase lack burst activity. Cell 1993; 74:621–631.

41 Steeghs K, Benders A, Oerlemans F, de Haan A, Heerschap A, Ruitenbeek W, et al. Altered Ca^{++} response in muscles with combined mitochondrial and cytosolic creatine kinase deficiencies. Cell 1997; 89:93–103.

42 Saupe KW, Spindler M, Tian R, Ingwall JS. Impaired cardiac energetics in mice lacking muscle-specific isoenzymes of creatine kinase. Circ Res 1998; 82:898-907.

43 Oliver CN, Starke-Reed PE, Stadtman R, Liu GJ, Carney JM, Fliyd RA. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion injury to gerbil brain. Proc Natl Acad Sci 1990; 87:5144-5147.

44 Burmistrov SO, Mashek OP, Kotin AM. The action of acute alcoholic intoxication on the antioxidant system and creatine kinase activity in the brain of rat embryos. Eksp Klin Farmakol 1992; 55:54-56.

45 Wolosker H, Panizzutti R, Englender S. Inhibition of creatine kinase by S-nitrosoglutathione. FEBS Lett 1996; 392:274-276.

46 Mekhfi H, Veksler V, Mateo P, Maupoil V, Rochette L, Ventura-Clapier R. Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils. Circ Res 1996; 78:1016-1027.

47 Stachowiak O, Dolder M, Wallimann T, Richter C. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J Biol Chem 1998; 273:16694-16699.

48 Nathan C and Xie QW. Nitric oxide synthases: roles, tools, and controls. Cell 1994; 78:915-918.

49 Yuan G, Kaneko M, Masuda H, Hon RB, Kobayashi A, Yamazaki N. Decrease in heart mitochondrial creatine kinase activity due to oxygen free radicals. Biochim Biophys Acta 1992; 1140:78-84

50 Meister A and Anderson ME. Glutathione. Annu Rev Biochem 1983; 52:711-760.

51 Lipskaya Tyu. Mitochondrial creatine kinase: Properties and function. Biochemistry (Mosc) 2001; 66:1361-1376.

52 Soboll S, Brdiczka D, Jahnke D, Schmidt A, Schlattner U, Wendt S, et al. Octamer-dimer transitions of mitochondrial creatine kinase in hear disease. J Mol Cell Cardiol 1999; 31:857-866.

53 Khuchua ZA, Qin W, Boero J, Cheng, J, Payne RM, Saks VA, et al. Octamer formation and coupling of cardiac sarcomeric mitochondrial creatine kinase are mediated by charged N-terminal residues. J Biol Chem 1998; 273:22990-22996.

54 Kenyon GL and Reed GH. Creatine kinase: structure-activity relationships. Adv Enzymol 1983; 54:367-426.

55 Furter R, Furter-Graves EM, Wallimann T. Creatine kinase: the reactive cysteine is required for synergism but is nonessential for catalysis. Biochemistry 1993; 32:7022-7029.

56 Zhou G, Somasyndaram T, Blanc E, Parthasarathy G, Ellingtin WR, Chapman MS. Transition state structure of arginine kinase: implications for catalysis of bimolecular reactions. Proc Natl Acad Sci USA 1998, 95:8449-8454.

57 Brown CS and Cunningham LW. Reaction of reactive sulfhydryl groups of creatine kinase with dansyl chloride. Biochemistry 1970; 9:3878-3885.

58 Reddy SR and Watts DC. Inhibition of creatine kinase by iodoalkanes. Further appraisal of the essential nature of the reactive thiol group. Biochim Biophys Acta 1979; 569:109-113.

III. 7. Efeito dos ácidos D-2-hidroxiglutárico e L-2-hidroxiglutárico sobre a atividade da Na$^+$,K$^+$-ATPase de membrana plasmática sináptica de ratos jovens

III. 7. 1. Reagentes

- Ácido D-2-hidroxiglutárico - Sigma Chemical CO (St. Louis, MO, USA)
- Ácido L-2-hidroxiglutárico - Sigma Chemical CO (St. Louis, MO, USA)
- Ácido clorídrico - Merck
- Ácido etilenodiaminotetracético (EDTA) - Merck
- Ácido N-2-hidroxietilpiperazina -N'-etano sulfônico (HEPES) - Sigma Chemical CO (St. Louis, MO, USA)
- Ácido orto-fosfórico - Merck
- Ácido tricloroacético - Reagen
- Adenosina - 5'- trifosfato (ATP) - Sigma Chemical CO (St. Louis, MO, USA)
- Albumina bovina - Sigma Chemical CO (St. Louis, MO, USA)
- Álcool polivinílico - Vetec
- Cloreto de magnésio - Sigma Chemical CO (St. Louis, MO, USA)
- Cloreto de potássio - Merck
- Coomasie Brilhante Blue G - Sigma Chemical CO (St. Louis, MO, USA)
- Molibdato de amônio - Vetec
- Ouabaína - Sigma Chemical CO (St. Louis, MO, USA)
- Sacarose - Reagen
- Trisma base - Sigma Chemical CO (St. Louis, MO, USA)
- Verde malaquita - Sigma Chemical CO (St. Louis, MO, USA)

Todos os reagentes utilizados foram de pureza analítica e as soluções foram preparadas em água destilada e deionizada pelo sistema Milli-Q.

III. 7. 2. Equipamentos e materiais permanentes

- Agitador de tubas Phoenix modelo AP 56 A
- Balança analítica Satorius
- Banho-maria De Leo tipo 697
- Centrífuga Eppendorf modelo 5403, rotor 16F12-17
- Centrífuga refrigerada Sorvall modelo 5-B rotor SS-34
- Cronômetro Citzen/Quartz modelo LWS 580 A
- Espectrofotômetro Milton Roy modelo Genesys 5
- Homogeneizador de vidro tipo Potter-Elvehjem
- Medidor de pH Orion, modelo 702
- Pipetas automática Gilson
- Ultracentrífuga Beckmann modelo L 80 rotor SW 28
- Vidrarias volumétricas

III. 7. 3. Animais experimentais

Foram utilizados ratos Wistar com 30 dias de idade, de ambos os sexos, provenientes do Biotério do Departamento de Bioquímica da UFRGS. Os animais foram criados em ninhadas de oito por gaiola e desmamados aos 21 dias de vida. Após o desmame, os ratos tinham livre acesso à água e alimentação padrão (ração comercial Guabi) e foram mantidos em ambiente climatizado (20°C ± 1°C) com ciclo claro-escuro de 12 horas.

III. 7. 4. Dissecação das estruturas cerebrais dos animais

Os animais foram mortos por decapitação. O escalpo foi rapidamente removido e o crânio aberto. O encéfalo foi imediatamente removido com auxílio de uma espátula e colocado sobre um papel filtro umedecido em meio de preparação (sacarose 0,32 M, HEPES 5mM e EDTA 0,1 mM, pH 7,5) sobre uma placa de Petri invertida sobre gelo picado. O córtex cerebral e o cerebelo foram dissecados, pesados, transferidos para um tubo de ensaio contendo o mesmo meio de preparação na proporção de 10 mL para cada 1g de tecido e mantidos em banho de gelo.

III. 7. 5. *Obtenção de membrana plasmática sináptica*

As membranas plasmáticas sinápticas foram isoladas conforme o método de Jones & Matus (1974). Para cada preparação foram utilizadas estruturas (córtex ou cerebelo) provenientes de dois animais. As estruturas previamente separadas foram homogeneizadas em Potter-Elvehjem a uma velocidade de aproximadamente 600 rpm. O homogeneizado foi submetido a uma centrifugação a 1.000 x g por 10 minutos a 4°C. O sedimento (SD1), contendo restos nucleares, células e membranas celulares foi descartado. O sobrenadante (SN1) foi centrifugado a 12.000 x g por 20 minutos a 4°C. O sobrenadante (SN2) foi desprezado e o sedimento (SN2) foi ressuspenso em solução hipotônica (Tris-HCl 5,0 mM, pH 8,1) e mantido em banho de gelo por 30 minutos, promovendo a lise do sinaptossoma.

Ao lisado foi acrescentada solução de sacarose 48% , resultando em uma mistura com concentração final de sacarose de 34% a qual foi aplicada em gradiente descontínuo de sacarose para a extração de membrana sináptica. O gradiente foi formado diretamente no tubo de centrifugação, pipetando 16 mL da mistura sacarose 34% (lisado), e sobre essa foi cuidadosamente acrescentada 16 mL de solução de sacarose 28,5% e uma terceira camada de 6 mL de sacarose 10%.

O gradiente assim obtido foi centrifugado a 60.000 x g por 110 minutos a 4°C. Dessa centrifugação, 3 frações foram obtidas. Conforme Jones & Matus (1974), a fração menos densa (F1) é composta basicamente por mielina; a fração F2 (entre as fases 28,5% e 34%) constituída principalmente por membranas plasmáticas sinápticas e o sedimento (F3), por mitocôndrias. Com pipeta automática, a fração F2 foi aspirada (aproximadamente 3,0 ml dessa fração) e suspensa em tampão Tris-HCl 5,0 mM, pH 8,1 e centrifugada a 37.000 x g por 20 minutos a 4°C, para remoção da sacarose residual. O sedimento resultante foi ressuspenso em meio de preparação (sacarose 0,32 M, HEPES 5 mM e EDTA 0,1 mM, pH 7,5) de modo a obter-se uma concentração final de proteínas de 0,15 a 0,25 mg/ml.

O material preparado foi mantido em banho de gelo durante todo o procedimento e foi armazenadas em alíquotas de 500 μL a -20°C, por um período máximo de 4 semanas. Esse material foi utilizado para os ensaios enzimáticos.

III. 7. 6. Determinação da atividade da Na$^+$,K$^+$-ATPase

A atividade medida conforme método de Tsakiris & Deliconstantinus (1984). O meio de reação padrão para o ensaio enzimático continha: cloreto de magnésio 5,0 mM; cloreto de sódio 80 mM; cloreto de potássio 20mM; Tris-HCl 40 mM pH 7,4; com ou sem ouabaína 1 mM (inibidor específico da Na$^+$,K$^+$-ATPase), em um volume final de 200 μL. Os ácidos testados foram dissolvidos em Tris-HCl pH 7,4 e adicionadas ao meio de incubação na concentração final de 1,0 mM.

A membrana plasmática sináptica foi adicionada ao meio de incubação em volume de 10 μL. O sistema foi pré-incubado durante 10 minutos a 37°C. A reação enzimática foi iniciada com a adição de ATP na concentração final de 3 mM. Decorridos 5 minutos de incubação, a reação foi interrompida com a adição de 200 μL de TCA 10% e o sistema foi colocado imediatamente em banho de gelo por um mínimo de 10 minutos. O fosfato inorgânico liberado foi medido segundo Chan *et al* (1986).

Em um segundo momento, as estruturas cerebrais foram homogeneizadas em meio de preparação (sacarose 0,32 M, HEPES 5mM e EDTA 0,1 mM, pH 7,5) e pré-incubadas por 1 hora em banho-maria a 37°C na presença ou ausência dos ácidos testados. Após, procedeu-se à preparação da membrana plasmática sináptica e a determinação da atividade enzimática conforme descrito.

Todos os experimentos foram feitos em duplicata. A atividade enzimática específica da Na$^+$,K$^+$-ATPase foi obtida pela diferença entre os sistemas com e sem ouabaína e foi expressa em nmol de fosfato inorgânico (Pi) liberado por minuto por miligrama de proteínas (nmol Pi. min^{-1}. mg proteína^{-1}).

III. 7. 7. Determinação de proteínas

A proteína foi determinada pelo método de Bradford (1976), utilizando-se albumina sérica bovina como padrão.

III. 7. 8. Análise estatística

Os dados foram analisados estatisticamente através da análise de variância de uma via seguida pelo teste de Duncan quando o valor de F era significativo. A análise estatística foi realizada através do programa SPSS-PC (Statistical Package for the Social Sciences).

III. 7. 9. Efeito dos ácidos D-2-hidroxiglutárico e L-2-hidroxiglutárico sobre a atividade da Na$^+$-K$^+$-ATPase de membrana plasmática sináptica de córtex cerebral de ratos jovens

Foi avaliado o efeito dos ácidos DGA e LGA, presentes no sistema de incubação na concentração de 1 mM, sobre a atividade da Na$^+$,K$^+$-ATPase de córtex cerebral (figura III.1.). Nessas condições experimentais, não foi observado qualquer efeito estatisticamente significante sobre a atividade da enzima [$F_{(2,17)} = 0,204$; $p=0,817$].
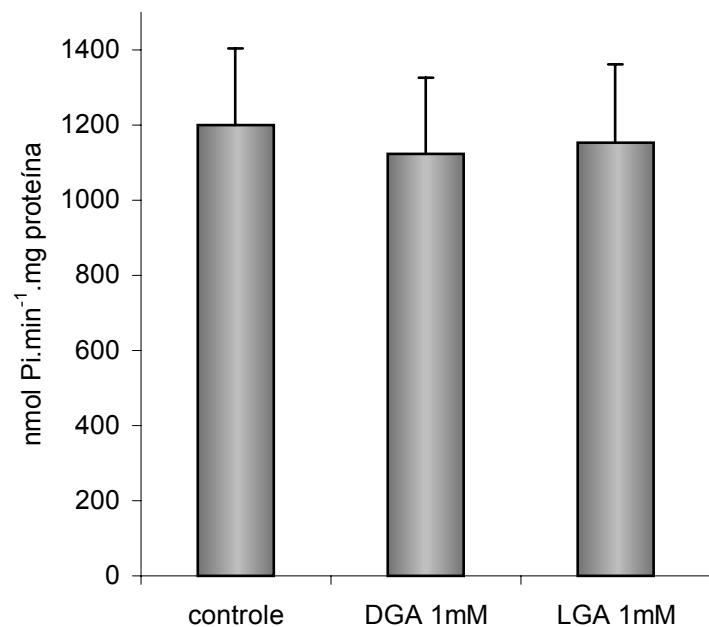
Figura III.1. Efeito do DGA e LGA sobre a atividade da Na$^+$,K$^+$-ATPase de membrana plasmática sináptica de córtex cerebral de ratos jovens.
Os resultados estão expressos como média ± desvio padrão para 6 experimentos independentes realizados em duplicata.

III. 7. 10. *Efeito dos ácidos D-2-hidroxiglutárico e L-2-hidroxiglutárico sobre a atividade da Na$^+$-K$^+$-ATPase de membrana plasmática sináptica de cerebelo de ratos jovens*

Foi avaliado o efeito dos ácidos DGA e LGA, presentes no sistema de incubação concentração de 1 mM, sobre a atividade da Na$^+$,K$^+$-ATPase de cerebelo (figura III.2.). Nessas condições experimentais, não foi observado qualquer efeito estatisticamente significante sobre a atividade da enzima [F(2,14) = 1,207; p=0,333].
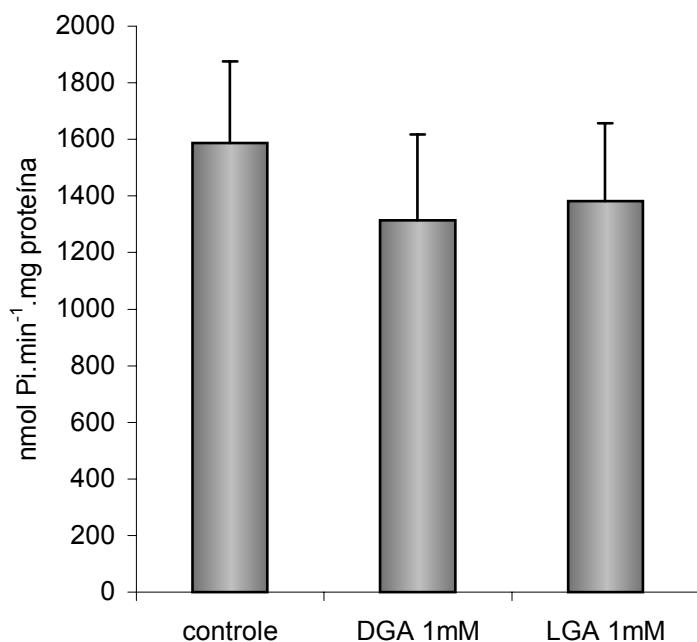
Figura III.2. Efeito do DGA e LGA sobre a atividade da Na$^+$,K$^+$-ATPase de membrana plasmática sináptica de cerebelo de ratos jovens.
Os resultados estão expressos como média ± desvio padrão para 5 experimentos independentes realizados em duplicata.

III. 7. 11. *Efeito da pré-incubação com os ácidos D-2-hidroxiglutárico e L-2-hidroxiglutárico sobre a atividade da Na^+-K^+-ATPase de membrana plasmática sináptica de córtex de ratos jovens*

Foi avaliado o efeito da pré-incubação de 1 hora com 1,0 mM dos ácidos DGA e LGA sobre a atividade da Na^+,K^+-ATPase de córtex cerebral (figura III.3.). Nessas condições experimentais, não foi observado qualquer efeito estatisticamente significante sobre a atividade da enzima [$F_{(2,11)}=0,044$, $p=0,957$].
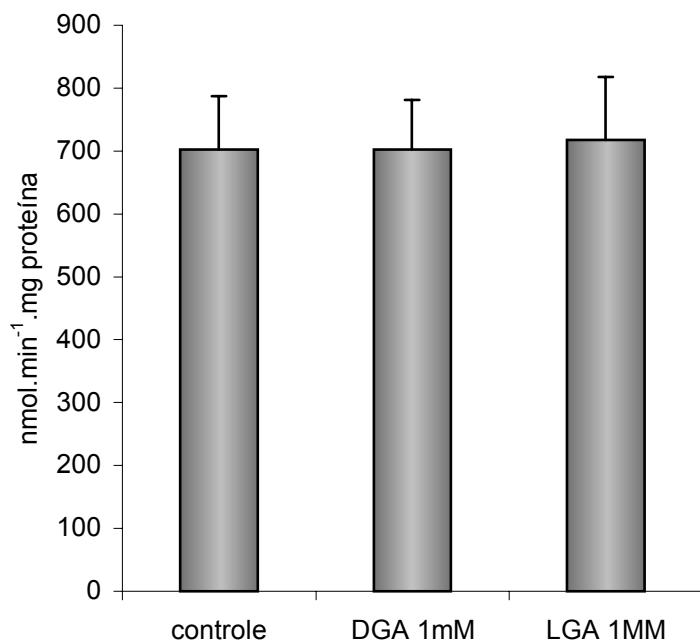
Figura III.3. Efeito a pré-incubação com DGA e LGA sobre a atividade da Na^+,K^+-ATPase de membrana plasmática sináptica de córtex cerebral de ratos jovens.
Os resultados estão expressos como média ± desvio padrão para 4 experimentos independentes realizados em duplicata.

III. 7. 12. *Efeito da pré-incubação com o ácido L-2-hidroxiglutárico sobre a atividade da Na⁺-K⁺-ATPase de membrana plasmática sináptica de cerebelo de ratos jovens*

Foi avaliado o efeito da pré-incubação de 1 hora com LGA 1,0 mM sobre a atividade da Na⁺,K⁺-ATPase de cerebelo (figura III.4.). Nessas condições experimentais, não foi observado qualquer efeito estatisticamente significante sobre a atividade da enzima [$t(6)=0,631$, $p=0,551$].
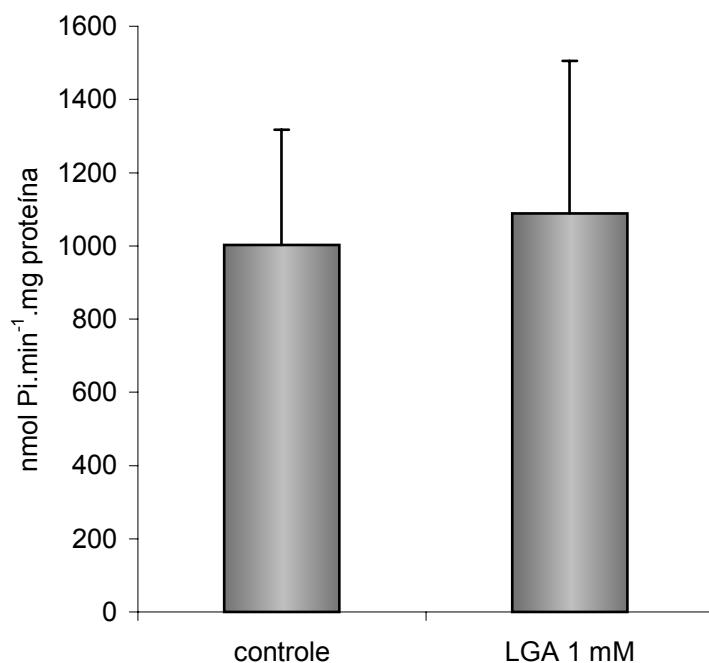

*Figura III.4.* Efeito da pré-incubação com LGA sobre a atividade da Na⁺,K⁺-ATPase de membrana plasmática sináptica de cerebelo de ratos jovens.

Os resultados estão expressos como média ± desvio padrão para 7 experimentos independentes realizados em duplicata.

IV. DISCUSSÃO

As acidúrias L-2-hidroxiglutárica (LHGA) e D-2-hidroxiglutárica (DHGA) são desordens metabólicas hereditárias bioquimicamente caracterizadas pelo acúmulo tecidual e elevada excreção urinária dos ácidos L-2-hidroxiglutárico (LGA) e D-2-hidroxiglutárico (DGA), respectivamente (Chalmers *et al*, 1980; Duran *et* al, 1980; Wanders *et al*, 1997; Barth *et al*, 1998; van der Knaap *et al*, 1999a,b).

Clinicamente, os pacientes com LHGA apresentam progressiva ataxia e deterioração mental, convulsões, sintomas piramidais e extrapiramidais e severa disfunção cerebelar. Também são relatados sintomas como tremores, movimentos coreiformes e prejuízo na fala, durante a infância e a adolescência. Exames de neuroimagem revelam atrofia cerebelar e alterações nos gânglios da base (Barth *et al*, 1992; Barth *et al*, 1993; Barth *et al*, 1998). Enquanto a sintomatologia clínica e o curso da doença apresentam-se de forma homogênea entre os pacientes com LHGA, a DHGA é caracterizada por pelo menos duas variantes: uma forma severa, cuja encefalopatia tem início precoce e um terço dos pacientes morre durante a infância, e uma outra variante com manifestações clínicas mais suaves. A maioria dos pacientes afetados apresenta retardo no desenvolvimento, retardo mental, hipotonia, miocardiopatia e convulsões. Ventriculomegalia, cistos subependimais e sinais de retardo na maturação cerebral são caracteristicamente observados à imagem de ressonância magnética (IMR). (Chalmers *et al*, 1980; van der Knaap *et al*, 1999a,b).

Embora muito pouco se saiba sobre os mecanismos envolvidos na fisiopatologia das disfunções teciduais nessas acidemias orgânicas, é possível que uma disfunção mitocondrial primária ou funcional possa estar relacionada, uma vez que, ao lado da elevada excreção urinária dos enantiômeros do ácido 2-hidroxiglutárico, grandes quantidades de lactato, α-cetoglutarato e outros intermediários do ciclo de Krebs são freqüentemente encontrados na urina de alguns pacientes com LHGA e DHGA (Hoffmann *et al*, 1995; Chen *et al*, 1996; Barth *et al*, 1998). Dessa forma, os tecidos cujo metabolismo energético seja mais dependente das funções oxidativas, como cérebro, coração e músculo esquelético (Sue *et al*, 1999) seriam preferencialmente afetados, como já tem sido demonstrado em um grande número de doenças mitocondriais (Anan *et al*, 1995; Bohlega *et al*, 1996).

Alterações no metabolismo energético celular são freqüentemente verificadas *in vitro*, através da avaliação da respiração mitocondrial em células inteiras ou mitocôndrias isoladas. Alternativamente, atividades enzimáticas isoladas podem ser determinadas. Nesse trabalho, foi investigada a influência *in vitro* de diferentes concentrações de DGA e LGA sobre vários parâmetros bioquímicos relacionados ao metabolismo energético celular nos

principais tecidos afetados nos pacientes com DHGA e LGHA: córtex cerebral, cerebelo, músculo esquelético e músculo cardíaco.

Primeiramente, foi observado que o DGA diminuiu a utilização de glicose *in vitro* por homogeneizado de córtex cerebral, o mesmo não ocorrendo em fatias. Por outro lado, a presença de LGA no meio de incubação não alterou esse parâmetro. Essa redução na utilização da glicose em homogeneizados pode refletir uma diminuição na atividade do Ciclo de Krebs. Além disso, a ausência do efeito do DGA nas fatias poderia ter ocorrido devido ao transporte limitado do ácido para o interior das células ou por falta de sensibilidade analítica do método utilizado. O próximo passo foi investigar a atividade do ciclo de Krebs através da medida da produção de CO_2 a partir de acetato radioativamente marcado. Uma significante redução na produção de CO_2 foi observada na presença de DGA no meio de incubação, tanto em homogeneizados quanto em fatias de córtex cerebral, enquanto o LGA não apresentou qualquer efeito significativo sobre esse parâmetro. Embora não se conheça como o DGA atravessa as membranas celulares, uma possível competição entre esse ácido e o acetato pelo mesmo sistema de transporte (carreador monocarboxílico) poderia reduzir a produção de CO_2 a partir desse substrato. Entretanto, redução semelhante na produção de CO_2 foi observada na presença de DGA utilizando-se citrato, um substrato que utiliza transportadores tricarboxilícos. Sendo assim, nossos dados indicam que o efeito do DGA sobre a produção de CO_2 seja, provavelmente, secundário a um bloqueio do Ciclo de Krebs e/ou da cadeia respiratória.

Nesse contexto, avaliamos o efeito *in vitro* de ambos os ácidos sobre a atividade dos complexos enzimáticos da cadeia respiratória em preparações de córtex cerebral e verificamos que o DGA inibiu significativamente (até 95%) a atividade da citocromo *c* oxidase (COX, complexo IV), enquanto as atividades dos demais complexos enzimáticos não foram afetadas pelo metabólito. O LGA não alterou a atividade de nenhum dos complexos enzimáticos estudados. Esses resultados demonstram que o DGA inibe seletivamente apenas um dos quatro complexos enzimáticos da cadeia respiratória, fundamental para a transferência de elétrons ao O_2, processo acoplado à fosforilação oxidativa e responsável pela maior parcela da energia (ATP) produzida nas células. Além disso, as observações de que tanto o LGA, o isômero óptico de DGA, bem como o ácido D-2-hidroximetilvalérico, um isômero D com massa molecular semelhante ao DGA, não alteraram a atividade da COX apontam para a especificidade do efeito inibitório exercido por esse composto.

A citocromo *c* oxidase (complexo IV) é uma proteína complexa multicomponente, embebida na membrana mitocondrial interna, composta por 13 subunidades, das quais 10 são codificadas pelo DNA nuclear e três pelo DNA mitocondrial. Ativa na forma dimérica em mamíferos, a COX é responsável pelo evento terminal na cadeia de transporte de elétrons no qual os elétrons provenientes do citocromo *c* são transferidos ao oxigênio molecular, reduzindo-o a água, enquanto um próton é bombeado para o espaço intermembranas.

O controle da fosforilação oxidativa é geralmente feito pelas concentrações de ADP e ATP. De acordo com a hipótese quimiosmótica (Nicholls & Ferguson, 1992; Arnold & Kadenbach; 1997; Arnold & Kadenbach 1999) a respiração mitocondrial é principalmente controlada pela força próton motriz (Δp) através da membrana mitocondrial interna. A estimulação do consumo de oxigênio por ADP em mitocôndrias isoladas tem sido explicada pela captação do ADP para o interior da mitocôndria, seguida da estimulação da ATP sintase e da conversão desse ADP em ATP, acompanhada pela diminuição do Δp. Essa redução no gradiente de prótons acaba por estimular a atividade das três bombas de prótons da cadeia respiratória (complexos I, III e IV) e, conseqüentemente, a respiração mitocondrial (Arnold & Kadenbach, 1999). Entretanto, um segundo mecanismo de controle da respiração celular, independente do Δp, tem sido descrito (Murphey & Brand, 1988; Papa *et al*, 1991; Kadenbach & Arnold, 1999). Esse mecanismo é baseado em uma inibição alostérica da atividade da citocromo *c* oxidase decorrente da troca do ADP ligado a um sítio de alta afinidade da enzima por ATP, na presença de altas razões ATP/ADP intramitocondriais, resultando na redução da respiração e do bombeamento de prótons através da membrana mitocondrial (Arnold & Kadenbach, 1997; Arnold & Kadenbach, 1999; Kadenbach & Arnold, 1999; Kadenbach *et al*, 2000). Recentemente, Kadenbach e colaboradores demonstraram uma fosforilação reversível e dependente de cAMP da COX com altos níveis de ATP. A pré-incubação de mitocôndrias na presença de cAMP, ATP e um sistema regenerador de ATP aumentou a inibição da COX induzida pelo ATP. Entretanto, a presença de Ca^{+2} na pré-incubação aboliu tal efeito inibitório do ATP. Dessa forma, têm sido postulada a existência *in vivo* de um equilíbrio entre a fosforilação da COX por proteínas quinases mitocondriais dependentes de cAMP e a defosforilação da enzima por proteínas fosfatases ativadas por Ca^{+2}. Em resumo, sob essas condições, a respiração mitocondrial seria regulada pela COX de acordo com a utilização do ATP, e também pelos substratos envolvidos (Kadenbach *et al*, 2000).

Sendo assim, poder-se-ia presumir que uma inibição da atividade da COX pelo DGA afetaria o controle da respiração mitocondrial, levando a uma redução na atividade do ciclo de Krebs e, conseqüentemente, ocasionando uma diminuição no consumo de glicose e na produção de CO_2 pelo córtex cerebral, conforme observado no presente estudo. Além disso, a elevação nos metabólitos do ciclo de Krebs encontrada em pacientes afetados por DHGA poderia ocorrer secundariamente ao bloqueio no fluxo de elétrons na cadeia respiratória causado por DGA, com conseqüente aumento nas concentrações de NADH e $FADH_2$. O aumento desses nucleotídeos reduzidos poderia levar a uma inibição da α-cetoglutarato desidrogenase e da succinato desidrogenase e conseqüente acúmulo de seus substratos, α-cetoglutarato e succinato. O lactato pode também estar aumentado pela elevada razão $NADH/NAD^+$. Por outro lado, é possível que a falta de energia causada pela inibição da atividade da COX poderia comprometer a síntese lipídica, como também a síntese de neurotransmissores a partir de intermediários do ciclo do ácido cítrico. Esse fato poderia estar relacionado a desmielinização e atrofia cerebral cortical observada nos pacientes com DHGA. Além disso, a deficiente produção de energia causada por DGA poderia causar a ativação de receptores NMDA, mesmo em concentrações normais de glutamato na fenda sináptica, por uma redução no potencial de membrana, secundário ao mau funcionamento da Na^+,K^+-ATPase, induzindo uma liberação dos canais voltagem-dependente bloqueados por Mg^{+2}, resultando no influxo de cálcio e, eventualmente, em morte celular (excitotoxicidade secundária).

Nesse contexto, é importante enfatizar que sintomas neuromusculares como hipotonia e miocardiopatia, comumente encontrados em pacientes com deficiência na atividade da COX, são freqüentemente observados em alguns pacientes com LHGA e DHGA. Além disso, a deficiência da atividade da COX causada por mutações que atingem genes que codificam a enzima também está associada à acidemia lática, podendo ocorrer em várias formas fenotípicas. A forma mais comum manifesta-se como síndrome de Leigh, entidade caracterizada por progressiva deterioração neurológica. Alguns pacientes com síndrome de Leigh apresentam uma redução de 80 – 90% na atividade da COX que afeta os tecidos com alta demanda energética (DiMauro *et al*, 1987; van Coster *et al*, 1991; Lombes *et al*, 1991; Robinson, 1993). Manifestações clínicas de outros distúrbios genéticos com deficiência de COX incluem a forma infantil fatal, na qual as crianças morrem por acidose lática durante o período neonatal e apresentam síndrome de Fanconi e miopatia (van Biervliet *et al*, 1977; DiMauro *et al*, 1980; Heiman-Patterson *et al*, 1982; Minchom *et al*, 1983; Miyabayashi *et al*, 1987), a forma reversível benigna (DiMauro *et*

al, 1983) e as formas nas quais os pacientes apresentam miocardiopatia (van Coster *et al*, 1991; Robinson *et al*, 1990; Glerum *et al*, 1989; Robinson *et al*, 1992).

Por outro lado, uma vez que os mecanismos responsáveis pelas alterações musculares e cardíacas presentes nessas patologias não são compreendidos, estendemos nossos estudos e decidimos investigar o efeito dos ácidos DGA e LGA sobre as atividades dos complexos enzimáticos componentes da cadeia respiratória em músculo esquelético e músculo cardíaco, na expectativa de determinar se esses ácidos poderiam comprometer o metabolismo energético nesses tecidos. O DGA significativamente inibiu a atividade da COX, de forma dose-dependente, em músculo esquelético e músculo cardíaco de ratos jovens, enquanto as atividades dos demais complexos da cadeia respiratória permaneceram inalteradas. Também verificamos que o DGA inibe a atividade da COX em homogeneizado de músculo esquelético humano.

Por outro lado, o LGA não alterou a atividade dos complexos enzimáticos estudados, de forma similar aos resultados obtidos em córtex cerebral. Esses achados estão de acordo com estudos anteriores que demonstraram que as atividades dos complexos I a IV da cadeia respiratória apresentam-se normais em biópsias de fígado e músculo esquelético de pacientes com LHGA (Barth *et al*, 1998).

A análise cinética do efeito do DGA sobre a COX demonstrou um perfil de inibição acompetitivo em relação ao citocromo *c*, nos três tecidos estudados, ou seja, o DGA é capaz de inibir a atividade da enzima após a formação do complexo enzima-substrato. Os valores de K_i obtidos no cérebro, músculo esquelético e músculo cardíaco de ratos jovens, ficaram na ordem de milimolar, semelhantes às concentrações encontradas nos líquidos biológicos dos pacientes com DHGA.

Estudos prévios têm demonstrado que inibidores como o óxido nítrico (> 1µM) e o peroxinitrito aumentam o K_m da COX para o O_2 por inibição competitiva irreversível com esse substrato (Brown & Cooper, 1994; Sharpe & Cooper, 1998). Dessa forma, para uma melhor elucidação dos mecanismos inibitórios do DGA sobre a COX seria interessante a avaliação de sua ação sobre a enzima em condições de pressão de O_2 controlada e próxima à fisiológica, bem como através das determinações de K_m e V_{max} e perfil inibitório do ácido com relação a esse substrato.

Relativamente ao grau de inibição da enzima causada pelo DGA e suas conseqüências sobre o metabolismo celular, a inibição variou de 45% a 90% em função das concentrações de DGA utilizadas no ensaio (0,5 – 5,0 mM) e do tecido em questão. Considerando que essa inibição possa ser extrapolada para a situação *in vivo*, poder-se-ia

questionar se esse grau de inibição comprometeria efetivamente o metabolismo energético. Alguns autores têm proposto que alterações na COX em músculo esquelético deveriam levar a manifestações fenotípicas somente se mais do que 70% da atividade enzimática fosse perdida (Letellier *et al*, 1994; Rossignol *et al*, 1999). No entanto, ratos deficientes em cobre, nos quais a atividade da COX está deficiente em torno de 50%, apresentam seu metabolismo energético muscular seriamente afetado (Kuznetsov *et al*, 1996; Tracey *et al*, 1997). Além disso, recentemente foi demonstrado que a atividade da COX é finamente regulada no músculo esquelético *in vivo,* com importantes implicações nas patologias mitocondriais (Kunz *et al*, 2000). Foi demonstrado que redução significante no fluxo respiratório é evidente mesmo em situações de moderada inibição da COX. Esses achados são corroborados por estudos que demonstraram uma razão lactato/piruvato aumentada e uma atividade da COX 35-45% dos valores normais em fibroblastos de pacientes cuja manifestação fenotípica da deficiência genética da COX ocorre principalmente como cardiomiopatia (Glerum *et al*, 1989; Robinson *et al*, 1990). Além disso, uma diminuição na atividade da COX semelhante a demonstrada em nossos estudos tem sido observada em tecido cerebral de pacientes com outras doenças neurodegenerativas como Doença de Huntington, Alzheimer e Parkinson (Wong-Riley, 1989).

No presente estudo, observamos uma inibição significativa da COX a partir de 0,5 mM de DGA, concentração semelhante aquela encontrada no plasma e líquor dos pacientes com DHGA (46-757 μm/L) (van der Knaap *et al*, 1999a,b). Embora as concentrações teciduais (cérebro e músculo esquelético e cardíaco) de DGA nesses pacientes não sejam ainda conhecidas, e tendo em vista que a DHGA é considerada uma acidemia orgânica cerebral, é possível que as concentrações intracelulares (onde o ácido é produzido) de DGA atinjam valores ainda mais elevados nessa desordem neurometabólica, como ocorre em outras acidemias orgânicas cerebrais cujos sintomas são predominantemente neurológicos (Hoffman *et al,* 1993). Portanto, se nossos resultados puderem ser extrapolados para a condição humana é possível que o DGA possa causar a uma redução no fluxo de elétrons da cadeia respiratória nos pacientes afetados por DHGA, provocando um déficit na produção de energia que poderia estar relacionado com a fisiopatologia das alterações neurodegenerativas e cardiomusculares apresentadas por esses pacientes.

Recentemente, Kölker e colaboradores demonstraram que DGA inibe a ATP sintase, reforçando a hipótese de que o DGA compromete o metabolismo energético celular. Essas alterações poderiam explicar a elevação de intermediários do ciclo de Krebs e de lactado no plasma e urina de alguns dos pacientes afetados por DHGA.

Por outro lado, a inibição da cadeia respiratória poderia prejudicar a habilidade das células em manter o potencial de membrana devido à depleção de ATP resultando em uma diminuição da atividade da Na^+, K^+-ATPase. (Erecinska & Dagani, 1990).

A Na^+,K^+-ATPase, enzima integral de membrana, é responsável pela restauração do equilíbrio entre os íons Na^+ e K^+ nas células, tanto antes com depois da passagem do impulso nervoso. A inibição da atividade da Na^+,K^+-ATPase está associada a diversas patologias, particularmente associadas a dano cerebral e a excitotoxicidade (Lees *et al*, 1991).

Avaliamos também os efeitos *in vitro* do DGA e do LGA sobre a atividade da Na^+,K^+-ATPase de membrana plasmática sináptica de córtex cerebral e cerebelo de ratos com 30 dias de vida para testar a influência direta dos ácidos sobre a enzima. Ambos os ácidos não causaram qualquer efeito significante sobre a atividade da enzima. Tais achados reforçam a ação seletiva do DGA sobre a COX.

Outro mecanismo celular importante na manutenção dos níveis constantes de ATP é o sistema catalisado pela creatina quinase (CK). Esse sistema tem papel fundamental na homeostase energética neuromuscular, sendo reconhecido como um importante regulador metabólico, devido a sua localização próxima aos sítios onde ocorrem a geração e o consumo de energia, garantindo a manutenção das concentrações de ATP necessárias a ATPases específicas e ao funcionamento normal das células. Em função disso, redução da atividade da CK tem sido relacionado ao desenvolvimento de diversos estados patológicos envolvendo os tecidos que apresentam elevada e flutuante necessidade energética, como o cérebro, o músculo esquelético e o músculo cardíaco. Uma diminuição na atividade da CK tem sido encontrada em diversas doenças neurodegenerativas, incluindo a Doença de Alzheymer, a Doença de Pick, epilepsia, esquizofrenia e psicose maníaco-depressiva. Além disso, suplementações de creatina e fosfocreatina têm demonstrado efeito neuroprotetor em casos de deficiência energética e na excitotoxicidade induzida por glutamato, provavelmente por promoverem um aumento nas concentrações de fosfato de alta energia no citosol das células neuronais.

Embora sintomas neuromusculares sejam comuns em doenças nas quais a atividade da CK está diminuída, muito pouco se sabe sobre a influência de metabólitos acumulados em doenças neurodegenerativas sobre sua atividade. Portanto, considerando nossos achados anteriores de inibição da cadeia respiratória pelo DGA e a estreita relação existente entre o sistema CK/PCr e a fosforilação oxidativa, estudamos o efeito *in vitro* dos ácidos DGA e LGA sobre a atividade total da CK (tCK), bem como das frações

citosólica (Cy-CK) e mitocondrial (Mi-CK) a partir de preparações de córtex cerebral, cerebelo, músculo esquelético e músculo cardíaco de ratos jovens.

Inicialmente observamos que o DGA inibiu significativamente a atividade da tCK em homogeneizado de córtex cerebral (30%) de modo dose-dependente. Também demonstramos que o DGA reduz significativamente a atividade da enzima tanto na fração mitocondrial (40%) quanto na citosólica (22%) em concentrações de 0,25 mM e superiores.

Posteriormente investigamos o efeito do DGA sobre as atividades da CK em cerebelo de ratos jovens, em concentrações semelhantes às testadas anteriormente em córtex cerebral, buscando determinar se o efeito do ácido sobre a CK era semelhante ao do córtex cerebral. Verificamos que o DGA inibiu a atividade da tCK cerebelar somente em concentrações de 2,5 mM e superiores. Além disso, o DGA inibiu seletivamente a isoforma mitocondrial, sem alterar a atividade da Cy-CK em cerebelo. Esses resultados demonstram que o cerebelo é muito menos sensível do que o córtex cerebral aos efeitos inibitórios mediados pelo DGA sobre a atividade da CK, visto que concentrações 10 vezes superiores foram necessárias para afetar a atividade da CK cerebelar (2,5 mM) com relação ao mesmo efeito observado no tecido cortical cerebral (0,25 mM). Nesse contexto, é importante enfatizar que as anormalidades cerebrais apresentadas pelos pacientes com DHGA são principalmente localizadas no córtex cerebral. Dessa forma, é possível presumir que a inibição diferencial desse importante sistema enzimático, necessário à manutenção da homeostasia energética cerebral, possa explicar, pelo menos em parte, a neuropatologia do DGA. Não sabemos porque a CK cerebelar é menos suscetível a ação inibitória do DGA. Entretanto, é possível que tais achados possam estar relacionados à distribuição das diferentes isoformas da enzima nas distintas regiões cerebrais. Nesse sentido, tem sido demonstrada a existência de diferentes subespécies da isoforma citosólica cerebral CK-B, chamadas Ba-CK e Bb-CK, as quais apresentam dimerização e distribuição tecido-específica. Portanto, em vista da distribuição distinta das subespécies de Cy-CK nas diferentes estruturas cerebrais, poder-se-ia presumir que o DGA poderia atuar predominantemente sobre isoformas da Cy-CK presentes no córtex cerebral e menos efetivamente sobre as isoformas presente no cerebelo.

Uma vez que os pacientes afetados por DHGA apresentam miopatia e cardiomiopatia, testamos o efeito do DGA sobre as isoenzimas de CK em músculo esquelético e músculo cardíaco de ratos. Demonstramos que o DGA inibe significativamente tanto a isoforma mitocondrial quanto a citosólica da CK em ambos os

tecidos. No entanto, observamos que o tecido cardíaco parece ser mais afetado do que o músculo esquelético, uma vez que, nesse tecido, a inibição da enzima ocorreu em concentrações de 0,25 mM do ácido enquanto no tecido muscular esquelético foram necessárias concentrações de 1,0 mM ou superiores. Tal fato poderia explicar porque uma parcela considerável dos pacientes com DHGA é acometida por cardiomiopatia que geralmente os leva a desenlace fatal.

A análise cinética do efeito do DGA sobre a atividade da tCK indicou um perfil de inibição não competitivo, com um K_i na ordem de milimolar em todos os tecidos estudados.

Verificamos também que o LGA não alterou a atividade da enzima em homogeneizados de córtex cerebral, músculo esquelético e cardíaco. Essa observação, aliada ao fato de que os ácidos α-cetoglutárico, glutárico e ácido D-hidroximetilvalérico, compostos com estrutura e massa molecular semelhantes ao DGA, não causaram qualquer alteração na atividade da enzima apontam para um efeito seletivo do DGA e não a um efeito inespecífico resultante de compostos ácidos, D-enantiômeros ou substâncias dicarboxílicas.

Em resumo, nossos achados demonstraram um importante bloqueio na cadeia respiratória e inibição significativa da atividade da CK, causada pelo DGA principalmente da isoforma mitocondrial, o que indica um distúrbio secundário no metabolismo energético mitocondrial causado pelo ácido. Uma vez que a Mi-CK ativa a transfosforilação entre ATP e creatina para ADP e PCr, uma redução na sua atividade poderia liberar menos ADP para ser recaptado ao interior da mitocôndria via ANT para ativação da OXPHOS. Ao mesmo tempo, menos PCr estaria disponível para tamponar a grande variação nos níveis de ATP intracelular, o que poderia se refletir em menor concentração de ATP disponível para suprir as necessidades celulares. Além disso, a inibição da isoforma citosólica pelo ácido agravaria o déficit energético, já que a transfosforilação entre a PCr e o ADP estaria reduzida..

Também demonstramos que o LGA inibe significativamente a atividade da creatina quinase em cerebelo e mais especificamente a isoforma mitocondrial, sem afetar a isoforma citosólica nesse tecido, bem como as atividades dessa enzima no córtex cerebral músculo esquelético e músculo cardíaco. Esses resultados apontam para um efeito seletivo do LGA sobre a CK cerebelar. Esse achado é extremamente interessante, uma vez que os pacientes com LHGA apresentam severa e progressiva deterioração dessa estrutura cerebral como característica neuropatológica mais relevante. Também demonstramos

através de estudos cinéticos que a inibição exercida pelo LGA sobre Mi-CK cerebelar apresenta um perfil não competitivo dose-dependente.

A Mi-CK é parte de um sistema único de tamponamento de energia, principalmente nos tecidos que apresentam elevada demanda energética, sendo também importante na inibição da abertura do poro de transição de permeabilidade mitocondrial (PTP) induzida por Ca^{+2} que leva à apoptose. Portanto, pode-se presumir que a inibição significativa da atividade da Mi-CK provocada por DGA predominantemente em córtex cerebral e por LGA em cerebelo, possa provocar um déficit no metabolismo energético cerebral, levando à apoptose celular e contribuindo, dessa forma, para explicar a atrofia cerebral característica dessas patologias.

O cérebro é muito sensível ao estresse oxidativo devido à sua alta taxa de consumo de oxigênio e energia, a quantidades relativamente baixas de antioxidantes e a grandes quantidades de ácidos graxos insaturados (Frantzeva *et al*, 2000). Sob condições em que a atividade da COX é bem controlada, a formação de espécies reativas de oxigênio (ROS) é pequena. Entretanto, quando a capacidade dessa enzima está reduzida, o risco de redução incompleta do oxigênio e de formação de ROS é aumentado (Staniek & Nhol, 2000). Portanto, a inibição significativa da atividade da COX causada pelo DGA poderia levar secundariamente ao estresse oxidativo. Nesse sentido, foi verificado recentemente que o DGA induz o estresse oxidativo, detectado através da utilização do corante fluorescente dihidrorodamina-123 em cultura primária de neurônios de pintos (Kölker *et* al, 2002). Além disso, estudos em nosso laboratório demonstraram recentemente que o DGA induz a lipoperoxidação em homogeneizado de córtex cerebral de ratos e reduz a reatividade das defesas antioxidantes do tecido (Latini *et* al, 2003). Portanto, o déficit energético e o estresse oxidativo poderiam ser mecanismos neurotóxicos causados pelo DGA.

Por outro lado, vários estudos têm mostrado que a Cy-CK e a Mi-CK tem sua atividade inibida por ROS e que o peroxinitrito (PN), produzido a partir do oxido nítrico (NO), oxida as cadeias laterais dos aminoácidos aromáticos e os grupamentos sulfidrila presentes na enzima (Konorev *et al*, 1998; Kaasik *et al*, 1999). Além disso, foi também demonstrado que a atividade da CK cerebral diminui após a exposição do tecido a radicais livres provavelmente por oxidação de resíduos de cisteína presentes na enzima.

Várias estratégias têm sido desenvolvidas para bloquear diferentes etapas da cascata de eventos que leva a injúria tecidual decorrente do estresse oxidativo. Entre elas, a prevenção da geração de ROS e sua neutralização por antioxidantes têm despertado especial interesse (Choi, 1990) tanto para a compreensão dos mecanismos protetores

fisiológicos como para fins terapêuticos. Sendo assim, buscamos investigar se a inibição da atividade da CK pelos ácidos DGA e LGA poderia ser mediada por ROS. Testamos a influência de vários antioxidantes sobre o efeito inibitório produzido pelo DGA sobre atividade da CK em córtex cerebral, cerebelo, músculo esquelético e músculo cardíaco de ratos, bem como sobre o efeito inibitório exercido pelo LGA sobre a Mi-CK em cerebelo. Verificamos que o glutatião reduzido (GSH), que fisiologicamente atua como um agente redutor de grupamentos tióis, preveniu totalmente o efeito inibitório exercido do DGA sobre a tCK em todos os tecidos estudados. Além disso, o GSH também preveniu, de forma semelhante, o efeito inibitório exercido LGA sobre a Mi-CK cerebelar. Por outro lado, α-tocoferol, um antioxidante endógeno bloqueador de radicais peroxila (ROO⁻) e capaz de interromper o processo de lipoperoxidação, o ácido ascórbico, que neutraliza radicais hidroxila e superóxido, e o L-NAME, um inibidor da oxido nítrico sintetase, não foram capazes de prevenir o efeito dos ácidos sobre a CK. Tais resultados sugerem que os ácidos não induzem a formação das espécies ativas de oxigênio e nitrogênio mais comuns e neutralizadas por esses antioxidantes.

A CK possui 8 grupamentos sulfidrila por monômero. Um resíduo altamente conservado de cisteína (Cys-283 na isoforma citosólica e Cys-278 na isoforma mitocondrial) está localizado próximo ao sítio catalítico da CK e é importante para a plena atividade enzimática. Acredita-se que esse resíduo interage com os grupamentos guanidino da creatina sendo responsável pela orientação correta do substrato no sítio ativo. Além disso, a forma octamérica da Mi-CK, estabilizada por grupamentos SH- (resíduos de cisteína) presentes nas regiões de contato entre os dímeros é tida como a forma ativa da enzima *in vivo*. Finalmente, a ligação do octâmero à membrana mitocondrial, necessária para a completa atividade biológica da enzima, também depende de grupamentos sulfidrila.

Esses grupamentos sulfidrila, em especial os resíduos próximos ao centro ativo, têm sido apontados como os principais alvos para a inativação química da CK por várias substâncias, não somente através de reações de oxidação como também por outras modificações pós-translacionais, como ocorre com o NO, via reações de *S*-nitrosilação, peroxinitrito (PN) pela formação de ácido sulfênico ou acrilamida por alquilação. Portanto, é possível que a inibição exercida por DGA e LGA possa ser mediada por uma modificação nos grupamentos sulfidrila necessários à sua função ou à manutenção da forma octamérica da enzima. Por outro lado, é conhecido que a modificação dos grupamentos tióis reativos leva a perda da atividade enzimática, mas não destrói a

habilidade de ligação ao substrato, fato que vai ao encontro de nossos achados que revelam um perfil de inibição não-competitivo causada pelos ácidos DGA e LGA sobre a enzima.

Gostaríamos de salientar que outros agentes neurotóxicos também modificaram afetar de forma distinta a atividade da CK em diferentes regiões cerebrais, apresentando maior efeito inibitório sobre enzima em córtex cerebral do que em cerebelo (Matsuoka *et al*, 1992; Hyakudo *et al*, 2001). Além disso, a ação inibitória desses compostos foi prevenida por agentes redutores como ditiotreitol e mercaptoetanol, indicando o envolvimento de grupamentos tióis nesse processo, fato semelhante ao que ocorreu em nosso estudo.

É difícil extrapolar nossos resultados experimentais realizados *in vitro* para as condições humanas (DHGA e LHGA). Contudo, se nossos resultados forem comprovados em estudos *in vivo*, é possível que as a inibição das isoformas de CK causada pelo DGA em concentrações similares às presentes nos tecidos e líquidos biológicos dos pacientes com DHGA possa estar implicada na fisiopatologia do severo quadro neurológico, muscular e cardíaco apresentado pelos pacientes com DHGA. Nesse particular, o desenvolvimento de modelos animais experimentais de DHGA e LHGA poderá nos responder se as alterações enzimáticas encontradas nesse trabalho também se refletem em um organismo íntegro. Caso tais resultados se reproduzam em modelos animais ou nas condições humanas, esses achados poderão ser de grande importância para a elucidação dos mecanismos fisiopatológicos envolvidos nas lesões teciduais presentes nos pacientes afetados por essas acidemias.

V. CONCLUSÕES

1. O ácido D-2-hidroxiglutárico inibiu significativamente o consumo de glicose *in vitro* por homogeneizado de córtex cerebral de ratos jovens.

2. O ácido D-2-hidroxiglutárico inibiu significativamente a produção de CO_2 *in vitro* a partir de acetato por homogeneizado e fatias de córtex cerebral de ratos jovens.

3. O ácido L-2-hidroxiglutárico não alterou o consumo de glicose e a produção de CO_2 em fatias de córtex cerebral de ratos jovens.

4. O ácido D-2-hidroxiglutárico inibiu significativamente *in vitro* a atividade da citocromo *c* oxidase (COX) de córtex cerebral de ratos jovens, sem alterar as atividades dos demais complexos da cadeia respiratória.

5. A inibição *in vitro* da atividade da COX em córtex cerebral de ratos pelo ácido D-2-hidroxiglutárico é do tipo acompetitiva, e o valor de Ki obtido foi na ordem de milimolar, semelhante aos valores encontrados no plasma e líquor dos pacientes com acidúria D-2-hidroxiglutárica (DHGA).

6. O ácido L-2-hidroxiglutárico não alterou a atividade de nenhum dos complexos enzimáticos da cadeia respiratória em preparações de córtex cerebral de ratos.

7. O ácido D-2-hidroxiglutárico inibiu significativamente *in vitro* a atividade das isoformas citosólica e mitocondrial da creatina quinase em preparações de córtex cerebral de ratos.

8. O ácido D-2-hidroxiglutárico inibiu significativamente *in vitro* a isoforma mitocondrial da creatina quinase em preparações de cerebelo de ratos, contudo o efeito inibitório foi observado em concentrações dez vezes superiores às obtidas em córtex cerebral.

9. O ácido L-2-hidróxiglutárico inibiu significativamente *in vitro* a atividade da creatina quinase mitocondrial em preparações de cerebelo de ratos jovens.

10. Estudos cinéticos demonstraram que ambos os ácidos (DGA e LGA) alteraram a atividade da creatina quinase através de um perfil de inibição não competitiva com a fosfocreatina, tanto em córtex cerebral quanto em cerebelo.

11. O ácido D-2-hidroxiglutárico inibiu significativamente a atividade das isoformas mitocondrial e citosólica da creatina quinase em preparações de músculo esquelético e cardíaco de ratos, sendo o tecido cardíaco mais sensível ao efeito inibitório do ácido.

12. O efeito inibitório exercido pelos ácidos DGA e LGA sobre a atividade da creatina quinase foi ser prevenido pela presença de glutatião reduzido no sistema de incubação, indicando um possível efeito dos ácidos sobre os grupamentos sulfidrila presentes na enzima.

13. Nenhum dos dois ácidos testados (DGA e LGA) alterou a atividade da Na^+,K^+-ATPase de membrana plasmática sináptica de córtex cerebral e/ou cerebelo de ratos jovens.

VI. PERSPECTIVAS

1. Determinar o efeito dos ácidos DGA e LGA sobre as concentrações de ATP, ADP, creatina e fosfocreatina em tecido cerebral e muscular de ratos.

2. Verificar o efeito dos ácidos DGA e LGA sobre a regulação da abertura induzida por Ca^{+2} dos poros de transição de permeabilidade mitocondriais em tecido cerebral de ratos.

3. Verificar o efeito dos ácidos DGA e LGA sobre a viabilidade celular em fatias de tecido cerebral, bem como em culturas primárias de astrócitos, neurônios e mistas.

4. Aprofundar os estudos sobre os mecanismos envolvidos na inibição da COX pelo DGA.

5. Verificar o efeito dos ácidos DGA e LGA sobre a oxidação dos grupamentos sulfidrila protéicos e não-protéicos em preparações de cérebro e tecido muscular de ratos jovens.

6. Determinar as concentrações de GSH após a exposição dos tecidos supracitados aos ácidos DGA e LGA.

7. Induzir um modelo animal para verificar o efeito in vivo dos metabólitos sobre vários parâmetros do metabolismo energético.

VII. BIBLIOGRAFIA COMPLEMENTAR

Abood LG and Geiger (1955) A. Breakdown of proteins and lipids during glucose-free perfusion of the cat's brain. Am. J. Physiol. 182: 557-560.

Babcock GT and Wikström M (1992) Oxygen activation and the conservation of energy in cell respiration. Nature 356: 301-309.

Barrientos A, Barros MH, Valnot I, Rötig A, Rustin P, Tzagoloff A (2002) Cytochrome oxidase in health and disease. Gene 286: 53-63.

Beal MF (1996) Mitochondria, free radicals, and neurodegeneration. Curr. Opin. Neurobiol. 6: 661-666.

Beutner G, Rück A, Riede B, Welte W, Brdiczka D (1996) Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. FEBS Lett. 396: 189-195.

Beutner G, Rück A, Riede B, Brdiczka D (1998) Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. Biochim. Biophys. Acta. 1368: 7-18.

Bittl JA and Ingwall JS (1985) Reaction rates of creatine kinase and ATP synthesis in the isolated rat heart. A 31P NMR magnetization transfer study. J. Biol. Chem. 260: 3512-3517.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantaties of protein utilizing the principle of protein-die binding. Anal. Biochem. 72: 248-254.

Brdiczka D and Wallimann T (1994) The importance of the outer mitochondrial compartment in regulation of energy metabolism. Mol. Cell. Biochem. 133/134: 69-83.

Brdiczka D, Knoll G, Riesinger I, Weiler U, Klug G, Benz R, Krause J (1986) in Advances in experimental medicine and biology. Vol 194. Myocardial and skeletal muscle bioenergetics (Brautbar N ed.) pp. 55-69. Plenum Press. New York.

Brooks SP and Suelter CH (1984) Arch. Biochem. Biophys. 257: 144-153.

Brown GC and Cooper CE (1994) Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal cytochrome oxidase respiration by competing with oxygen at cytochrome oxidase. FEBS Lett. 356: 295-298.

Burbaeva GSh, Aksenova MV, Bibikova VI (1987) BB creatine phosphokinase activity and content of the brain structures in senile dementia and Alzheimer's diseases. Zh. Nevropatol. Psikhiatr. Im. S. S. Korsakova. 87:1374-1378.

Burbaeva GSh, Kalinenko OO, Aksenova MB (1990) Decreased level of immunoreactive creatine phosphokinase BB isoenzymes in the brain of patients with schizophrenia and senile dementia of the Alzheimer type. Zh. Nevropatol. Psikhiatr. Im. S. S. Korsakova. 90: 85-87.

Burton, B.K. (1987) Inborn Errors of Metabolism: the clinical diagnosis in early infancy. Pediatrics 79:359.

Camargo Neto E, Schulte J, Silva LCS, Giugliani R (1993) Cromatografia em camada delgada para a detecção neonatal de fenilcetonúria e outras aminoacidopatias. Rev. Bras. Anál. Clín. 25: 81-82.

Capaldi RA (1992) Structure and function of cytochrome oxidase. Ann. Rev. Biochem. 59: 569-596.

Cataldo AM and Broadwell RD (1986) Cytochemical identification of cerebral glycogen and glucose-6-phosphate activity under normal and experimental conditions. I. Neurons and glia. J. Electron. Microsc. Techn. 3: 413-437.

Chan K, Delfert D, Junger KD (1986) A direst clorimetric assay for Ca2+-stimulated ATPase activity. Analytical Biochemistry 157: 375-380.

Choi DW (1990) Methods for antagonizing glutamate neurotoxicity, Cerebrovasc. Brain Metab. Rev. 2: 105–147.

Cunnigham VJ, Hargreaves RJ, Pelling D and Moorhouse SR (1986) Regional blood-brain glucose transfer in the rat : A novel double-membrane kinetic analysis. J. Cereb. Blood Flow Metab. 6: 305-314.

De Klerk LB, Huijmans JG, Stroink H, Robben SG, Jacobs C, Duran M. (1997) L-2-hydroxyglutaric aciduria: clinical heterogeneity versus biochemical homogeneity in a sibship. Neuropediatrics 28: 314-317.

Di Donato S (2000) Disorders related to mitochondrial membranes: pathology of the respiratory chain and neurodegeneration. J. Inherit. Metab. Dis. 23: 247-263.

DiMauro S, Servidei S, Zeviani M, DiRocco M, DeVivo DC, DiDonato S, Uziel G, Berry K, Hoganson G, Johnsen SD, et al. (1987) Cytochrome c oxidase deficiency in Leigh syndrome. Ann. Neurol. 22: 498-506.

DiMauro S, Nicholson JF, Hays AP, Eastwood AB, Papadimitriou A, Koenigsberger R, DeVivo DC. (1983) Benign infantile mitochondrial myopathy due to reversible cytochrome c oxidase deficiency. Ann Neurol. 14: 226-234.

Diogo L, Fineza I, Canha J, Borges L, Cardoso ML, Vilarinho L . (1996) Macrocephaly as the presenting feature of L-2-hydroxyglutaric aciduria in a 5-month old boy. J. Inherit. Metab. Dis. 19: 369-370.

Eppenberger HM, Dawsin DM, Kaplan NO (1967) The comparative enzymology of creatine kinases. I. Isolation and characterization from chicken and rabbit tissues. J. Biol. Chem. 242: 204-209.

Erecinska M and Silver IA (1994) Ions and energy in mammalian brain. Progress in Neurobiology 43: 37-71.

Frantseva MV, Perez Velazquez JL, Tsoraklidis G, Mendonca AJ, Adamchik Y, Mills LR, Carlen PL, Burnham MW (2000) Oxidative stress is involved in seizure-induced neurodegeneration in the kindling model of epilepsy, Neuroscience 97: 431–435.

Friedman DL and Perryman MB (1991) Compartmentation of brain-type creatine kinase and ubiquitous mitochondrial creatine kinase in neurons: evidence for a creatine phosphate energy shuttle in adult rat brain. J. Comp. Neurol. 343: 500-511.

Garrod AE (1908) Inborns errors of metabolism Lancet 2:1.

Gerring K (1990): Subunit assembly and funtional maturation of Na, K-ATPase. J. Membranme. Biol. 155: 109-121.

Gibson KM, Craigen WJ, Herman GE, Jakobs C. (1993) D-2-Hydroxyglutaric aciduria in a newborn with neurological abnormalities: a new neurometabolic disorder? J. Inherit. Metab. Dis. 16: 497-500.

Gibson KM, ten Brink HJ, Schor DSM, Kok RM, Bootsma AH, Hoffmann GF, Jackobs C. (1993) Stable-isotope dilution analysis of D- and L-2-hydroxyglutaric acids: application to the detection of D- and L-2-hydroxyglutaric acidemias. Pediatr. Res. 34: 277-280.

Gibson QH (1948) Biochem. J. 42: 13-18.

Greene DA (1983) Metabolic abnormalities in diabetic peripheral nerve: Relation to impaired function . Metabolism 32: 118-123.

Hawkins RA, Miller AL, Cremer JE, Veech RL (1974) Measurement of the rate of glucose utilization by rat brain in vivo. J. Neurochem. 23: 917-923.

Heiman-Patterson TD, Bonilla E, DiMauro S, Foreman J, Schotland DL. Cytochrome-c-oxidase deficiency in a floppy infant. Neurology. 1982 Aug;32(8):898-901.

Hoffmann GF (1994) Selective screening for inborn errors of metabolism – past, present and future. Eur. J. Pediatr. 153 (Suppl. 1): S2-S8.

Hoffmann GF, Gibson KM, Trefz FK, Nyhan WL, Bremer HJ, Rating D (1994) Neurological manifestations of organic acid disorders. Eur. J. Pediatr. 153 (suppl.1): S94-S100.

Holtzman D, Tsuji M, Wallimann T, Hemmer (1993) Functional maturation of creatine kinase. Dev. Neurosci. 15: 261-270.

Hornemann T, Rutishauser D, Wallimann T (2000) Why is creatine kinase a dimer? Evidence for cooperativity between the two subunits. Biochim Biophys Acta. 1480: 365-373.

Jacobs H, Heldt HW, Klingenberg M (1964) High activity of creatine kinase in mitochondria from muscle and brain and evidence for a separate mitochondrial isoenzyme of creatine kinase. Biochem. Biophys. Res. Commun 16: 516-521.

Jacobson IR, Hagberg H, Sandberg M, Hanberger A (1986) Ouabain-induced changes in estracellularaspartate, glutamate and GABA levels in the rabbit olfactory bulb in vivo. Neurosci. Lett 64: 211-215.

Jansen GA and Wanders RJ (1993) L-2-hydroxyglutarate dehydrogenase: identification of a novel enzyme activity in rat and human liver. Implications for L-2-hydroxyglutaric acidemia. Biochim. Biophys. Acta 1225: 53-56.

Kaasik A, Minajeva A, De Souza, Ventura-Clapier R, Veksler V (1999) Nitric oxide inhibts cardiac energy prodution via inhibition of mitochondrial creatine kinase. FEBS Lett. 444: 75-77.

Kanemitsu F, Kawanishi I, Mizushima J (1982) Characteristics of mitochondrial creatine kinases from normal human heart and liver tissues. Clin. Chim. Acta 119: 307-317.

Kaufman EE, Nelson T, Fales HM, Levin DM (1988) Isolation and characterization of a hydroxyacid-oxoacid transhydrogenase from rat kidney mitochondria. J. Biol. Chem. 263: 16872-16879.

Konorev EA, Hogg N, Kalyanaraman B (1998) Rapid and irreversible inhibition of creatine kinase by peroxynitrite. FEBS Lett. 427: 171-174.

Korge P, Byrd SK and Campbell KB (1993) Functional coupling between sarcoplasmic-reticulum-bound creatine kinase and Ca(2+)-ATPase. Eur. J. Biochem. 213: 973-980.

Krause SM and Jacobus WE (1992) Specific enhancement of the cardiac myofibrillar ATPase by bound creatine kinase. J. Biol. Chem. 267: 2480-2486.

Kuzhikandathil EV and Molloy GR (1994) Transcription of the brain creatine kinase gene in glial cells is modulated by cyclic AMP-dependent protein kinase. J. Neurosci. Res. 39: 70-72.

Kuznetsov AV, Clark JF, Winkler K, Kunz WS (1996) Increase of flux control of cytochrome c oxidase in copper-deficient mottled brindled mice. J. Biol. Chem. 271: 283-288.

Lajtha A and Toth J (1961) The brain barrier system: II. Uptake and transport of amino acids by the brain. J. Neurochem. 8: 216-225.

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. 2002 Nov 29;956(2):367-73.

Less GJ, Lehmann A, Sandberg M, Hamberger A (1990) The neurotoxicity of ouabain, a sodium –potassium ATPase inhibition, in the rat hippocampus. Neurosc. Lett. 120: 159-162.

Lingrel JB and Kuntzweiler T (1994) Na+,K+-ATPase. J. Biochem. Chem. 269: 196599-196662.

Lombes A, Nakase H, Tritschler HJ, Kadenbach B, Bonilla E, DeVivo DC, Schon EA, DiMauro S (1991) Biochemical and molecular analysis of cytochrome c oxidase deficiency in Leigh's syndrome. Neurology 41:491-8.

Lund-Andersen H (1979) Transport of glucose from blood to brain. Physiol. Rev. 59: 305-352.

Manson GF, Behar KL, Rothman DL and Shulman RG (1992) NMR determination of intracerebral glucose concentration and transport kinetics in rat brain. J. Cereb. Blood Flow Metab. 12: 448-445.

Medina JM. The role of lactate as an energy substrate for the brain during the early neonatal period. Biol. Neonate. 48(237-244).

Miyabayashi S, Ito T, Abukawa D, Narisawa K, Tada K, Tanaka M, Ozawa T, Droste M, Kadenbach B. (1987) Immunochemical study in three patients with cytochrome c oxidase deficiency presenting Leigh's encephalomyelopathy. J. Inherit. Metab. Dis. 10: 289-292.

Murphey MP and Brand MD (1988) The stoichiometry of charge translocation by cytochrome oxidase and the cytochrome bc1 complex of mitochondria at high membrane potential. Eur. J. Biochem. 173: 645-651.

Owen OE, Morgan AP, Kemp HG, Sullivan JM, Herrera MG, Cahill GF Jr (1967) Brain metabolism during fasting. J. Clin. Invest. 46: 1589-1595.

Papa S, Capitanio N, Capitanio G, De Nitto E, Minuto M (1991) The cytochrome chain of mitochondria exhibits variable H+/e- stoichiometry. FEBS Lett. 288: 183-186.

Pinto ALR, Raymond KM, Bruck I, Antoniuk AS (1998) Estudo de Prevalência em Recém-Nascidos por Deficiência de Biotinidase. Rev. Saúde Pública 32: 148-152.

Pollitt RJ et al - Neonatal screening for inborn errors of metabolism: cost, yeld and outcome. Health Technol Assess, 1 (7):1-202, 1997.

Poyton RO and McEwen JE (1996) Crosstalk between nuclear and mitochondrial genomes. Annu. Rev. Biochem. 65: 563-607.

Rapport RL, Harris AB, Friel PN, Ojemann GA (1975) Human epilaptic brain. Na+-K+-ATPase activity and phanytoin concentration. Arch. Neurol. 33: 549-554.

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

Robinson BH, Chow W, Petrova-Benedict R, Clarke JT, Van Allen MI, Becker LE, Boulton JE, Ragan I. Fatal combined defects in mitochondrial multienzyme complexes in two siblings. Eur J Pediatr. 1992 May;151(5):347-52.

Robinson BH (1993) Lactic acidemia. Biochim Biophys Acta. 1182: 231-234.

Robinson BH, Glerum DM, Chow W, Petrova-Benedict R, Lightowlers R, Capaldi R (1990) The use of skin fibroblasts cultures in the detection of respiratory chain defects in patients with lacticacidemia. Pediatr. Res. 28: 549-555.

Rossi AM, Eppenberger HM, Volpe P, Cotrufo R. Wallimann T (1990) Muscle-type MM creatine kinase is specifically bound to sarcoplasmic reticulum and can support Ca2+ uptake and regulate local ATP/ADP ratios. J. Biol. Chem. 265: 5258-5266.

Roth GS, Joseph JA, Mason RP (1995) Membrane alterations as causes of impaired signal transduction in Alzheimer's disease and aging. Trends Neurosci. 18: 203-206.

Saraste M (1990) Structural features of cytochrome c oxidase. Q. Rev. Biophys. 23: 331-366.

Saraste M (1999) Oxidative phosphorilation at the fin de siècle. Science 283: 1488-1492.

Schapira AH. Mitochondrial disorders: an overview. J Bioenerg Biomembr. 1997 Apr;29(2):105-7.

Schapira AHV (1999) Mitochondrial disorders. Biochim. Biophys. Acta. 1410: 99-102.

Schlattner U, Forstner M, Eder M, Stachowiak O, Fritz-Wolf K, Wallimann T (1998) Functional aspects of the X-ray structure of mitochondrial creatine kinase: a molecular physiology approach. Mol. Cell. Biochem. 184: 125-140.

Schmidt BJ, Martins AM, Fisberg RM, Müller R, Adell ACA, Subero EM (1987) Fenilquetonuria. Aspectos clínicos y terapéuticos. Pediatria al Dia 3: 257-256.

Schneider C, Stull GA, Apple FS (1988) Kinetic characterization of human heart and skeletal muscle CK isoenzymes. Enzyme 39: 220-226.

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

Sewell AC, Heil M, Podebrad F, Mosandl A (1998) Chiral compounds in metabolism: a look in the molecular mirror. Eur. J. Pediatr. 157: 185-191.

Sharpe MA and Cooper CE (1998) Interaction o peroxinitrite with mitochondrial cytochrome oxidase: catalytic production of nitric oxide and irreversible inhibition of enzyme activity. J. Biol. Chem. 273: 30961-30972.

Siesjo BK. In: Brain energy metabolism, Wiley, New York, 1978.

Sinclair LA (1982) A new look at the inborn errors of metabolism. Ann. Clin. Biochem. 19: 314-321.

Skou JC and Esmann M (1992) The Na,K-ATPase. Journal of Bioenergetics and Biomembranes. 24: 249-261.

Spitzer JJ (1973) CNS and fatty acid metabolism. The Physiologist 16: 55-58.

Staniek K, Nohl H (2000) Are mitochondria a permanent source of reactive oxygen species? Biochim. Biophys. Acta 1460: 268–275.

Stein W, Bohner J, Steinhart R, Eggstein M (1982) Macro creatine kinase: determination and differentiation of two types by their activation energies. Clin. Chem. 28: 19-24.

Tracey I, Dunn JF, Radda GK (1997) A 31P-magnetic resonance spectroscopy and biochemical study of the mo(vbr) mouse: potential model for the mitochondrial encephalomyopathies. Muscle Nerve 20: 1352-1359.

Tubbs PK and Greville GD (1961) The oxidation of D-α-hydroxyacids in animal tissues. Biochem. J. 81: 104-114.

Van Biervliet JP, Bruinvis L, Ketting D, De Bree PK, Van der Heiden C, Wadman SK. (1977) Hereditary mitochondrial myopathy with lactic acidemia, a De Toni-Fanconi-Debre syndrome, and a defective respiratory chain in voluntary striated muscles. Pediatr Res. 11: 1088-93.

Ventura-Clapier R, Veksler V (1994) Myocardial ischemic contracture. Metabolites affect rigor tension development and stiffness. Circ. Res. 74: 920-929.

Voet D and Voet JG (1995) Biochemistry. Second edition. John Wiley & sons, Inc. New York.

Wajner M, Wannmacher CMD, Gaidzinski D, Dutra-Filho CS (1986) Detection of inborn errors of metabolism in patients of paediatric intensive care units of Porto Alegre, Brazil. Comparison between the prevalence of such disturbances in a selected and an unselected sample. Brazilian Journal of Genetics IX: 331-340.

Wallace DC (1999) Mitochondrial diseases in man and mouse. Science 283: 1482-1487.

Wallimann T, Schlosser T, Eppenberger HM (1984) Function of M-line-bound creatine kinase as intramyofibrillar ATP regenerator and the receiving end of the phoshporylcreatine shuttle in muscle. J. Biol. Chem. 259: 5238-2546.

Wallimann T, Sclösser T, Eppenberger HM (1984) Function of M-line-bound creatine kinase as intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle. J. Biol. Chem. 259: 5238-5246.

Wallimann T, Walzthony D, Wegmann G, Moser H, Eppenberger HM, Barrantes FJ (1985) Subcellular localization of creatine kinase in Torpedo electrocytes: Association with acetylcholine receptor-rich membranes J. Cell. Biol. 100: 1063-1072.

Wappner RS (1993) Biochemical diagnosis of genetic diseases. Pediatr. Ann. 22: 282-292, 295-297.

Wilcken B, Pitt J, Heath D, Walsh P, Wilson G, Buchanan N. (1993) L-2-hydroxyglutaric aciduria: three Australian cases. J. Inherit. Metab. Dis. 16: 501-504.

Wong-Riley MTT (1989) Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. TINS 12: 94-101

Zafeiriou DI, Sewell A, Savvopoulou-Augoustidou P, Gombakis N, Katzos G (2001) L-2-hydroxyglutaric aciduria presenting as status epileticus. Brain & Dev. 23: 255-257.

Zeviani M and Antozzi C (1997) Mitochondrial disorders. Mol. Hum. Reprod. 3: 133-148.

Zilles K, Qu M, Schleicher A, Schroeter M, Kramer M and Witte OW (1995) Plasticity and neurotransmitter receptor changes in Alzheimer's disease and experimental cortical infarcts. Arzneim-Forsch 45: 361-366.