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EFEITOS DOS PRINCIPAIS ÁCIDOS GRAXOS ACUMULADOS NA DEFICIÊNCIA DA DESIDROGENASE DE ACIL-COA DE CADEIA MÉDIA SOBRE A HOMEOSTASE ENERGÉTICA MITOCONDRIAL E PARÂMETROS DE ESTRESSE OXIDATIVO EM CÉREBRO DE RATOS JOVENS

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Aos meus pais, melhores pais do mundo.

“Não é a inteligência nem a ciência que salvam o homem: é o amor.”

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Introdução e Objetivos

RESUMO

A deficiência da desidrogenase de acil-CoA de cadeia média (MCAD) é o mais frequente defeito de oxidação de ácidos graxos, caracterizado bioquimicamente pelo acúmulo tecidual predominante dos ácidos graxos de cadeia média octanoato (AO), decanoato (AD) e *cis*-4-decenoato (AcD). Embora os sinais clínicos dos afetados sejam fundamentalmente neurológicos, os mecanismos fisiopatológicos do dano do sistema nervoso central apresentado pelos pacientes afetados por esse distúrbio ainda não estão esclarecidos. Tem sido, no entanto, levantada a hipótese de que os ácidos graxos acumulados nesta doença possam exercer efeitos tóxicos. Neste cenário, o objetivo do presente trabalho foi investigar os efeitos *in vitro* dos ácidos AO, AD e AcD sobre parâmetros de função mitocondrial e de estresse oxidativo em cérebro de ratos de 30 dias de vida, uma vez que o metabolismo energético é muito ativo e as defesas antioxidantes estão diminuídas neste tecido. Inicialmente, observamos que todos os ácidos graxos testados dimuíram o potencial de membrana mitocondrial em preparações mitocondriais de cérebro de ratos, sendo que as concentrações mais altas testadas do AD e do AcD exerceram efeitos comparáveis ao de um clássico desacoplador da fosforilação oxidativa, sugerindo que estes ácidos graxos atuem como desacopladores. Os ácidos AO, AD e AcD também aumentaram o estado 4 da respiração mitocondrial e diminuíram os valores do índice de controle respiratório. Além disso, o AD e o AcD diminuíram o estado 3 da respiração tanto com glutamato/malato quanto com succinato como substratos, enquanto que o AO dimiuiu o mesmo parâmetro apenas com o succinato. Também encontramos uma diminuição na razão ADP/O na presença de AD e AcD. O atractilosídeo, inibidor do translocador de nucleotídeos de adenina (ANT), foi capaz de impedir parcialmente o efeito desacoplador do AD, sem alterar os efeitos do AO e do AcD, sugerindo um envolvimento deste translocador no efeito desacoplador do AD. O AO e o AD também diminuíram o consumo de oxigênio em mitocôndrias desacopladas. O AcD também diminuiu a produção de peróxido de hidrogênio, sendo seu efeito maior do que o da rotenona, que inibe o fluxo reverso de elétrons. Os AO e AD diminuíram as atividades dos complexos I-III e IV da cadeia transportadora de elétrons, e o AD também inibiu a atividade do complexo II-III. Todos os três ácidos graxos testados diminuíram o conteúdo de equivalentes reduzidos de NAD(P)H na matriz mitocondrial e a posterior adição de rotenona apenas reverteu o efeito do AO, sugerindo que estes equivalentes estejam sendo perdidos para o meio extramitocondrial. Os ácidos AD e AcD também induziram um maior inchamento mitocondrial, sugerindo que estes ácidos graxos possam atuar como desacopladores provavelmente devido a uma permeabilização não-seletiva da membrana mitocondrial interna.

Também avaliamos o efeito dos mesmos ácidos graxos sobre parâmetros de estresse oxidativo. Observamos que o AO, o AD e o AcD induziram um aumento na peroxidação lipídica em córtex cerebral de ratos, evidenciado por um aumento nos níveis de substâncias reativas ao ácido tiobarbitúrico (TBA-RS) e na medida de quimioluminescência espontânea. Os AD e AcD também causaram dano oxidativo proteico, visto que aumentaram o conteúdo de carbonilas e diminuíram o conteúdo de grupamentos sulfidrila. Além disso, todos os ácidos

graxos testados diminuíram as defesas antioxidantes não-enzimáticas, evidenciado pela diminuição do potencial antioxidante total do tecido (TRAP). Entretanto apenas o AD e o AcD diminuíram os níveis de glutationa reduzida (GSH), o principal antioxidante não-enzimático presente no cérebro. O AcD não ocasionou um aumento nos níveis de TBA-RS em preparações mitocondriais de cérebro de ratos, sugerindo que outros componenentes celulares sejam essenciais para os efeitos pró-oxidantes deste ácido graxo. Entretanto, não foi observado nenhum efeito do AD sobre níveis de GSH e o conteúdo de grupamentos sulfidrila em preparações citosólicas, sugerindo um papel para a mitocôndria nos efeitos causados por este ácido graxo.

Devemos também enfatizar que os efeitos mais pronunciados foram verificados pelo AcD, seguido do AD e por último do AO. Estes resultados, tomados em seu conjunto, indicam que os principais metabólitos acumulados na deficiência de MCAD exercem efeitos neurotóxicos importantes. Dessa forma, é possível que uma disfunção mitocondrial e o estresse oxidativo, possivelmente com outros mecanismos, atuem sinergicamente, colaborando para o dano neurológico apresentado pelos pacientes afetados pela deficiência da MCAD.

ABSTRACT

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (MCADD) is the most frequent fatty acid oxidation disorder, leading to the accumulation of octanoic (AO), decanoic (AD) and *cis*-4-decenoic (AcD) acids. Considering that the pathophisiology of the neurological damage found in MCAD-deficient patients is not clear yet, and that a role for neurotoxic accumulating metabolites has been considered, the aim of the present work was to investigate the *in vitro* effect of AO, AD and AcD on mitochondrial function and oxidative stress parameters in 30-day-old rat brain. Initially we investigated the effect of these fatty acids on mitochondrial homeostasis, and we found that all these three metabolites diminished the mitochondrial membrane potential in mitochondrial preparations of rat brain, and the effects of the highest concentration of AD and AcD were similar to the CCCP effect, a classical uncoupler, suggesting that these fatty acids act as uncouplers of oxidative phosphorylation. AO, AD and AcD also increased state 4 respiration and decreased respiratory control ratio. AD and AcD also decreased state 3 respiration with either glutamate/malate or succinate as substrates, while AO only decreased in the presence of succinate. We also observed a decrease in the ADP/O ratio caused by AD and AcD. Atractyloside, an inhibitor of adenine nucleotide transporter (ANT), mildly prevented the uncoupler effect of AD, without any effect of AcD- and AO-induced effects, suggesting a role for ANT on AD-induced effects. AO and AD also diminished uncoupled state (state U) in mitochondrial preparations. AcD also decreased hydrogen peroxide production, and this effect was greater than rotenone effect, which inhibits the reverse electron flow. AO and AD inhibited the activities of the respiratory chain complexes I-III and IV, and AD also inhibited complex II-III activity. All the fatty acids tested decreased mitochondrial matrix NAD(P)H content, and the further addition of rotenone only reverted AO-induced effect, indicating that these reduced equivalents might be lost from the mitochondrial matrix. AD and AcD also induced a mitochondrial swelling, suggesting that these fatty acids act as uncouplers probably due to a non-selective mitochondrial inner-membrane. We also evaluated the effect of the same fatty acids on oxidative stress parameters. We first observed that lipid peroxidation was increased in the presence of AO, AD and AcD, as observed by the increase in the thiobarbituric acid-reactive substances (TBA-RS) levels and spontaneous chemiluminescence. AD and AcD also caused protein oxidative damage, since they increased carbonyl content and decreased sulfhydryl group content. All the fatty acids tested also decreased the non-enzymatic antioxidant defenses, as they decreased the total radical-trapping antioxidant potential (TRAP). However, only AD and AcD decreased reduced glutathione (GSH) levels, the major antioxidant in the cell. AcD did not induce an increase in TBA-RS levels in mitochondrial preparations, suggesting that mitochondria were not involved in AcD-induced effects. Furthermore, AD did not alter GSH levels and sulfhydryl group content in cytosolic preparations, suggesting a role for mitochondria in AD-induced effects. Taken together, these results suggest that the MCADD accumulating fatty acids cause important neurotoxic effects. Thus, it is feasible that a mitochondrial dysfunction and oxidative stress, allied to other possible toxic effects, act

synergistically, collaborating to the neurological damage found in MCAD-deficient patients.

LISTA DE ABREVIATURAS

$\Delta\Psi m$ – potencial de membrana mitocondrial

8-OHdGA – 8-hidroxi-2'-deoxiguanosina

AcD – ácido *cis*-4-decenóico

AD – ácido decanóico

ANT – translocador de nucleotídeos de adenina

AO – ácido octanóico

ATC – atractilosídeo

CAT – catalase

CCCP – cianeto de carbonila de meta-clorofenil-hidrazona

CoA – coenzima A

CoQ – coenzima Q

CsA – ciclosporina A

DCFH – 2',7'-di-hidroclorofluoresceína

EIM – erros inatos do metabolismo

ERN – espécies reativas de nitrogênio

ERO – espécies reativas de oxigênio

GSH – glutationa reduzida

LCHAD – desidrogenase de 3-hidroxi-acil-CoA de cadeia longa

LDL – lipoproteína de baixa densidade

MCAD – desidrogenase de acil-CoA de cadeia média

MEL – melatonina

Pi – fosfato inorgânico

RCR – índice de controle respiratório

SCAD – desidrogenase de acil-CoA de cadeia curta

SCHAD – desidrogenase de 3-hidroxi-acil-CoA de cadeia curta

SOD – superóxido dismutase

TAR – reatividade antioxidante total do tecido

TBA-RS – substâncias reativas ao ácido tiobarbitúrico

TRAP - potencial antioxidante total do tecido

TRO – trolox

VLCAD – desidrogenase de acil-CoA de cadeia muito longa

I.1. INTRODUÇÃO

I.1.1. Erros Inatos do Metabolismo

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

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

essas doenças em seu conjunto afetam aproximadamente 1 a cada 500/2.000 recém nascidos vivos (Baric, Furnic e Hoffmann, 2001).

I.1.2. Defeitos de Oxidação de Ácidos Graxos

A beta-oxidação mitocondrial de ácidos graxos é a principal fonte de energia para a síntese de ATP, principalmente em períodos de jejum. Este processo gera acetil-coenzima A e energia na forma de ATP. A rota de oxidação dos ácidos graxos é complexa e inclui muitos passos: captação celular de ácidos graxos, ativação desses mesmos ácidos graxos a ésteres acil-CoA, trans-esterificação a acilcarnitinas, translocação através da membrana mitocondrial, re-esterificação a acil-CoA, e a espiral da beta-oxidação intramitocondrial, que fornece elétrons para flavoproteínas transferidoras de elétrons e acetil-CoA. Cada etapa da espiral de oxidação é catalisada por enzimas específicas para o comprimento da cadeia carbônica do ácido graxo (Smith, Marks e Lieberman, 2005).

Na década de setenta foram descritos os primeiros erros inatos do metabolismo atribuídos a defeitos na oxidação dos ácidos graxos em pacientes com astenia ou rabdomiólise induzida por exercício. Pouco tempo depois foi descrita a deficiência sistêmica de carnitina e, em 1982, a deficiência da desidrogenase de acil-CoA de cadeia média (MCAD) foi diagnosticada em pacientes que apresentavam descompensação metabólica com sintomas neurológicos durante o jejum (Kolvraa et al., 1982). Atualmente, já estão descritas pelo menos 23 diferentes entidades clínicas dentro deste grupo de doenças, incluindo defeitos no transporte de carnitina na membrana plasmática, nas enzimas carnitina palmitoiltransferase I e II, carnitina/acilcarnitina translocase, nas

desidrogenases de acil-CoA de cadeia muito longa (VLCAD), média (MCAD) e curta (SCAD), na 2,4-dienoil-CoA redutase e nas desidrogenases de 3-hidroxi-acil-CoA de cadeia longa (LCHAD) e curta (SCHAD), bem como na proteína trifuncional mitocondrial (Roe e Ding, 2001).

Acredita-se que a prevalência dessas doenças seja subestimada, visto que seu diagnóstico depende da detecção dos metabólitos acumulados por métodos sofisticados e equipamentos de alto custo que poucos laboratórios possuem (Walker, 1994). Além disso, devido à semelhança do quadro clínico, uma parte considerável dos pacientes afetados por defeitos de oxidação de ácidos graxos é diagnosticada erroneamente como síndrome de morte súbita da infância, infecção bacteriana aguda (septicemia), síndrome de Reye, fígado gorduroso da gravidez ou síndrome de vômito cíclico (Rinaldo et al., 1998).

Dentre os defeitos de beta-oxidação, a deficiência de MCAD é o mais frequente destes distúrbios, com uma prevalência similar à da fenilcetonúria, o mais frequente erro inato do metabolismo.

I.1.3. Deficiência de Desidrogenase de Acil-CoA de Cadeia Média (MCAD)

A deficiência de MCAD é um defeito hereditário metabólico de herança autossômica recessiva cujos primeiros sinais clínico-laboratoriais geralmente aparecem entre os primeiros dias de vida até os seis anos de idade (Wilcken et al., 2007), podendo, no entanto, ocorrer na fase adulta (Ruitenbeek et al., 1995). Os pacientes afetados pela deficiência de MCAD geralmente são assintomáticos e os sintomas são precipitados por períodos de jejum ou de outras formas de estresse metabólico, usualmente associadas a infecções virais ou bacterianas ou mesmo à

vacinação (Derks et al., 2006). Pacientes afetados pela deficiência de MCAD apresentam uma sintomatologia muito variada, incluindo episódios de vômitos, letargia, apnéia e coma, podendo levar à morte súbita (Grosse et al., 2006). Podem também apresentar atraso no desenvolvimento psicomotor, rabdomiólise, paralisia cerebral, retardo no crescimento, problemas comportamentais e déficit de atenção (Roe e Ding, 2001). Durante as crises mais graves o coma, muitas vezes acompanhado de convulsões, e a hiperamonemia com disfunção hepática são frequentes (Ruitenbeek et al., 1995).

Os principais achados neuropatológicos dos pacientes com deficiência de MCAD são microcefalia, edema cerebral e anomalias no lobo frontal (Egidio et al., 1989; Maegawa et al., 2008). Em muitos casos ocorre acúmulo microvesicular de lipídeos no fígado, similar ao acúmulo observado em pacientes com síndrome de Reye (Roe e Ding, 2001). Também pode ocorrer rabdomiólise, acúmulo microvesicular de lipídeos entre as miofibrilas e de glicogênio detectados por biópsia muscular (Ruitenbeek et al., 1995).

Se não diagnosticados precocemente, 20 a 25% dos pacientes afetados pela deficiência de MCAD morrem durante o primeiro episódio de crise metabólica (Grosse et al., 2006), enquanto o risco de sofrer uma crise fatal estende-se por toda a vida (Roe e Ding, 2001).

I.1.3.1. Metabólitos Acumulados nos Pacientes com Deficiência de MCAD

O defeito no catabolismo de ácidos graxos de cadeia média ocasiona o acúmulo destes ácidos graxos e seus derivados que, principalmente durante os períodos de crise, encontram-se muito aumentados no sangue e em outros

tecidos, bem como na urina dos pacientes (Tabela I). Os principais metabólitos acumulados são os ácidos graxos de cadeia média octanoato, decanoato e *cis*-4-decenoato.

Tabela I**:** Concentrações dos principais metabólitos acumulados em pacientes com deficiência de MCAD comparados com indivíduos normais.

Metabólito	**Indivíduos normais**	**Pacientes com deficiência de MCAD**
No sangue (em μM)		
ácido octanóico[b]	0-15	22- 672
ácido decanóico[b]	5-43	2-228
ácido *cis*-4- decenóico[b]	0-2	34-515
octanoilcarnitina[c]	<0,22	0,33-28,3
hexanoilcarnitina[c]	<0,21	0,13-4
decanoilcarnitina[c]	<0,30	0,11-2,6
decenoilcarnitina[c]	<0,04	0,13-1,7
Na urina (em μg/mg de creatinina[1] ou μmol/mmol creatinina[2])		
hexanoilglicina[1,d]	0,21-1,9	3,1-653
fenilpropionilglicina[1,d]	0-1,1	1,2-180
suberilglicina[1,c]	0-95	13-4553
ácido adípico[2,a]	-----	1,5-150
ácido subérico[2,a]	-----	0-214

(Adaptado de: [a]Downing et al., 1989, [b]Onkenhout et al., 1995; [c]Chace et al., 1997; [d]Rinaldo et al., 1998;).

Dentre os metabólitos acumulados, o ácido *cis*-4-decenóico, derivado do ácido linoleico, é considerado o composto patognomônico da deficiência de MCAD. A concentração plasmática desta substância em pessoas normais varia entre 0-2 μmol/L, enquanto que nos pacientes sintomáticos varia entre 34-515 μmol/L (Onkenhout et al., 1995). Outro achado comum na doença é a elevação da concentração urinária de hexanoilglicina, fenilpropionilglicina e suberilglicina (Costa et al., 2000).

I.1.3.2. Aspectos Moleculares da Deficiência de MCAD

A deficiência de MCAD é uma doença autossômica recessiva, cuja mutação mais comum é uma mutação ponto que resulta na substituição de lisina por ácido glutâmico no aminoácido 329 do precursor da MCAD (K329E). Isso ocorre devido à adição de um nucleotídeo adenina (na posição 985) no lugar de guanina no gene da MCAD (c.985A>G), mutação responsável por aproximadamente 80-90% dos alelos mutantes em caucasianos. Deve-se considerar também que o desenvolvimento dos sintomas deve ser influenciado pelo grau de estresse metabólico e a combinação de fatores, tais como infecção e jejum (Matsubara et al., 1992). Por outro lado, mais recentemente tem-se tentado correlacionar o tipo de mutação envolvida na doença com o acúmulo de metabólitos nos pacientes, sendo que aparentemente a presença da mutação c.985A>G parece estar correlacionada a maiores níveis de acúmulo neonatal de octanoilcarnitina no plasma bem como de acilglicinas na urina. Além disso, as mutações c.985A>G e c.583G>A parecem resultar em maior severidade, enquanto que a mutação

c.199T>C está associada a sintomas menos severos da doença (Waddell et al., 2006).

I.1.3.3. Tratamento da Deficiência de MCAD

O principal objetivo do tratamento da deficiência de MCAD é prevenir o desenvolvimento de retardo mental com anormalidades cerebrais e morte súbita (Kompare e Rizzo, 2008). A terapia aplicada aos pacientes com deficiência de MCAD consiste em proporcionar uma ingestão adequada de calorias, evitar o jejum e outras situações de catabolismo e ter cuidados especiais durante episódios de infecções (Roe e Ding, 2001).

A L-carnitina tem sido administrada em pacientes com doenças metabólicas com acúmulo de ácidos orgânicos, tais como acidemia propiônica, acidemia metilmalônica, acidemia isovalérica, acidemia glutárica tipo II, síndrome de Reye e também em portadores de deficiência de MCAD. A L-carnitina liga-se a ácidos orgânicos permitindo sua excreção na forma de acilcarnitinas. No caso da deficiência de MCAD, ocorre a conversão de octanoato a octanoilcarnitina, substância que se acredita ser de menor toxicidade e de excreção mais fácil do que o octanoato. Portanto, não deve ser surpreendente o fato de se observar níveis plasmáticos baixos de L-carnitina em portadores de deficiência de MCAD durante as crises (Kim et al., 1984, 1990). Resultados iniciais têm demonstrado que o tratamento dos pacientes por 4 semanas com L-carnitina evita a queda dos níveis de carnitina plasmática e aumenta a excreção urinária de acilcarnitinas, além de aumentar a tolerância dos pacientes ao exercício (Lee et al., 2005).

I.1.3.4. Fisiopatologia na Deficiência de MCAD

A síntese diminuída de corpos cetônicos durante o jejum é uma característica da deficiência de MCAD, fazendo com que aumente a importância da glicose sanguínea como fonte de energia celular, ocasionando hipoglicemia nos pacientes. Outra consequência da não-utilização dos ácidos graxos de cadeia média na síntese de corpos cetônicos é o acúmulo de acil-CoA de ácido graxo de cadeia média dentro das mitocôndrias. Eleva-se então a razão acil-CoA:CoA, causando a inibição das enzimas piruvato desidrogenase e α-cetoglutarato desidrogenase, que utilizam coenzima A como substrato. Assim, ocorre diminuição da conversão do piruvato a acetil-CoA e diminuição na velocidade do ciclo do ácido cítrico, visto que a síntese do citrato e a conversão do α-cetoglutarato a sucinil-CoA também estão diminuídas. Além disso, a sucinil-CoA ligase é inibida pelo ácido octanóico e também por intermediários de acil-CoA. Com a baixa produção de acetil-CoA, há diminuição da síntese de citrato. O citrato, por sua vez, é precursor de malato, substância necessária para a produção de glicose, via gliconeogênese, e precursor de malonil-CoA, o principal regulador inibitório da CPTI, enzima responsável pela entrada de ácidos graxos de cadeia longa na mitocôndria. Portanto, a diminuição dos níveis de citrato ocasionada pelo acúmulo do octanoato e outros ácidos graxos na deficiência de MCAD provoca também uma diminuição da gliconeogênese e um aumento da entrada de ácidos graxos de cadeia longa na mitocôndria, o que deve ser um agravante para a hipoglicemia e deve provocar o acúmulo de derivados de acil-CoA graxos nos pacientes (Roe e Ding, 2001).

Por outro lado, acredita-se que o quadro de letargia, que pode evoluir a coma e morte, seja devido, particularmente, ao acúmulo de ácidos graxos de cadeia média tóxicos e seus derivados (Gregersen et al., 2008). Até o presente momento, poucos estudos foram realizados sobre os efeitos tóxicos dos ácidos graxos de cadeia média acumulados na deficiência de MCAD. Foi demonstrado que o octanoato causa um aumento do consumo de O_2 e produção de CO_2, sem causar um correspondente aumento na produção de ATP em fígado de ratos (Berry et al., 1983; Scholz et al., 1984). Além deste, outros efeitos têm sido atribuídos ao octanoato, quando testado *in vitro*, como inibição do controle do volume de astrócitos e da Na^+,K^+-ATPase em cultura de células gliais (Olson et al., 1989), que poderiam estar relacionados ao edema cerebral observado na deficiência de MCAD e também na síndrome de Reye, ambas caracterizadas por acúmulo de octanoato nos tecidos dos pacientes afetados. Foi também demonstrado que o octanoato e o decanoato são inibidores do transporte de ácidos orgânicos através do plexo coróide em coelhos. Os autores do trabalho sugeriram que tal efeito poderia impedir a depuração do octanoato e compostos relacionados, contribuindo para o acúmulo desses compostos no cérebro e no líquido cerebroespinhal e para a encefalopatia das doenças em que essas substâncias se acumulam (Kim et al., 1983).

Mais recentemente, foi demonstrado que os ácidos octanóico, decanóico e *cis*-4decenóico inibem *in vitro* importantes parâmetros do metabolismo energético em cérebro de ratos jovens, incluindo as atividades de complexos da cadeia respiratória, da creatina quinase e Na^+,K^+-ATPase, bem como a produção de

$^{14}CO_2$ a partir de [U-^{14}C]glicose, [1-^{14}C]acetato e [U-^{14}C] citrato (de Assis et al., 2003, 2006; Reis de Assis et al., 2004).

I.1.4. Modelos Animais de Doenças em que há Acúmulo de Ácidos Graxos

O primeiro modelo animal com acúmulo de octanoato foi obtido em coelhos, através da infusão deste ácido graxo com o objetivo de simular sintomas da síndrome de Reye (Trauner e Huttenlocher, 1978; Trauner, 1982). Os animais injetados com octanoato apresentaram algumas das anormalidades clínicas e bioquímicas observadas em pacientes com esta encefalopatia hepática, como hiperventilação, hiperamonemia e depósito de gordura no fígado. Algumas alterações foram também observadas no cérebro dos coelhos. Posteriormente, foi descrita a inibição da atividade da Na^+,K^+-ATPase em diferentes regiões cerebrais dos animais injetados com octanoato. Tal efeito passou a ser considerado como um possível mecanismo causador da encefalopatia observada na síndrome de Reye, uma vez que as concentrações plasmática e cerebral de octanoato atingida nos coelhos foram similares àquelas encontradas nos pacientes com a doença (Trauner, 1980).

Foram também investigados os efeitos da infusão aguda de octanoato em camundongos sobre as concentrações cerebrais de alguns compostos envolvidos com o metabolismo energético, quando os animais estavam nos estados de pré-coma ou coma. Alterações nas concentrações de glicose, glicogênio, ATP e fosfocreatina foram encontradas apenas na formação reticular do cérebro dos animais, enquanto as concentrações destas mesmas substâncias em outras estruturas cerebrais foram normais (McCandless, 1985).

Em outros estudos, realizados com o intuito de mimetizar um defeito na oxidação de ácidos graxos, foram empregados inibidores específicos das desidrogenases de acil-CoA *in vivo*, como os ácidos espiropentaneacético (Tserng et al., 1991) e 2-mercaptoacetato em ratos (Bauché et al., 1981) ou o 2-mercaptoacetato em camundongos (Del Prete et al., 1998). Além disso, outros estudos utilizaram derivados da 2-alquinoil-CoA como inibidores *in vitro* de MCAD extraída de rim de porcos (Freund et al., 1985).

Recentemente, Derks e colaboradores (2008) desenvolveram um modelo animal em camundongos de deficiência de MCAD quimicamente induzido pela administração aguda de ácido 2-tetradecilglicídico, um inibidor da oxidação mitocondrial de ácidos graxos, o qual foi utilizado para investigar alterações no metabolismo da glicose no fígado dos animais (Derks et al., 2008).

I.1.5. Fosforilação Oxidativa e Homeostase Mitocondrial

A fosforilação oxidativa é o processo pelo qual o O_2 é reduzido a H_2O, por elétrons doados pelo NADH e $FADH_2$, que fluem através de vários pares redox (cadeia transportadora de elétrons), gerando ATP a partir de ADP e Fosfato inorgânico (Pi) (Nelson e Cox, 2000). Em eucariotos, a fosforilação oxidativa ocorre nas mitocôndrias, mais especificamente na cadeia respiratória, e é responsável pela maior parte da energia produzida pela célula. As mitocôndrias são corpúsculos envoltos por uma membrana externa, facilmente permeável a pequenas moléculas e íons, e por uma membrana interna, impermeável à maioria das moléculas e íons, incluindo prótons (Nelson e Cox, 2000). A membrana interna contém transportadores específicos para a passagem de substâncias

como o piruvato, glicerolfosfato, malato, ácidos graxos e outras moléculas essenciais às funções mitocondriais (Abeles, Frey e Jencks, 1992). O fluxo de elétrons do NADH e $FADH_2$ até o O_2 se dá através de complexos enzimáticos ancorados na membrana mitocondrial interna (cadeia transportadora de elétrons). Essa transferência de elétrons é impulsionada por um crescente potencial redox existente entre os equivalentes reduzidos (NADH e o $FADH_2$), os complexos enzimáticos da cadeia transportadora de elétrons e o O_2, que é o aceptor final dessa cadeia de reações de oxidação.

A cadeia respiratória é composta por vários complexos enzimáticos e uma coenzima lipossolúvel, a coenzima Q (CoQ) ou ubiquinona (Di Donato, 2000). O complexo I, conhecido como NADH desidrogenase ou NADH: ubiquinona oxidorredutase, transfere os elétrons do NADH para a ubiquinona. O complexo II (succinato desidrogenase) reduz a ubiquinona com elétrons do $FADH_2$ provenientes da oxidação do succinato a fumarato no ciclo do ácido cítrico. O complexo III, citocromo bc_1 ou ubiquinona-citocromo *c* oxidoredutase, catalisa a redução do citocromo *c* a partir da ubiquinona reduzida. Na parte final da cadeia de transporte de elétrons, o complexo IV (citocromo *c* oxidase) catalisa a transferência de elétrons de moléculas reduzidas de citocromo *c* para O_2, formando H_2O. São necessárias quatro moléculas de citocromo *c* para reduzir completamente uma molécula de O_2. Todos esses complexos possuem grupamentos prostéticos específicos para desempenharem o papel de aceptores e doadores de elétrons (Abeles, Frey e Jencks, 1992). No entanto, a membrana mitocondrial interna é impermeável às moléculas de NADH ou a $FADH_2$, necessitando sistemas de transferência desses equivalentes reduzidos do citosol

para a matriz mitocondrial. Nesse contexto, a oxidação do NADH formado no citosol é possibilitada por sistemas chamados lançadeiras, que transferem elétrons do NADH do citosol para a matriz, através de moléculas capazes de ser transportadas através da membrana mitocondrial interna. Existem duas lançadeiras para este fim, designadas de lançadeira do glicerol-3-fosfato e lançadeira de malato/aspartato, conforme ilustra a figura 1. Uma vez formadas na matriz, as moléculas de NADH e $FADH_2$ podem ceder elétrons para o complexo I ou para a CoQ, respectivamente, suprindo a cadeia respiratória (Nelson e Cox, 2000).

O fluxo de elétrons através dos complexos da cadeia respiratória é acompanhado pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembranas, através dos complexos I, III e IV, gerando um potencial de membrana. Assim, cria-se um gradiente eletroquímico transmembrana que pode ser utilizado por um quinto complexo protéico, a ATP sintase, para a síntese de ATP. Dessa forma, a oxidação de substratos energéticos está acoplada ao processo de fosforilação do ADP, ou seja, quando o potencial de membrana é dissipado pelo fluxo de prótons a favor do gradiente eletroquímico, a energia liberada é utilizada pela ATP sintase, que atua como uma bomba de prótons ATP-dependente (Nelson e Cox, 2000).

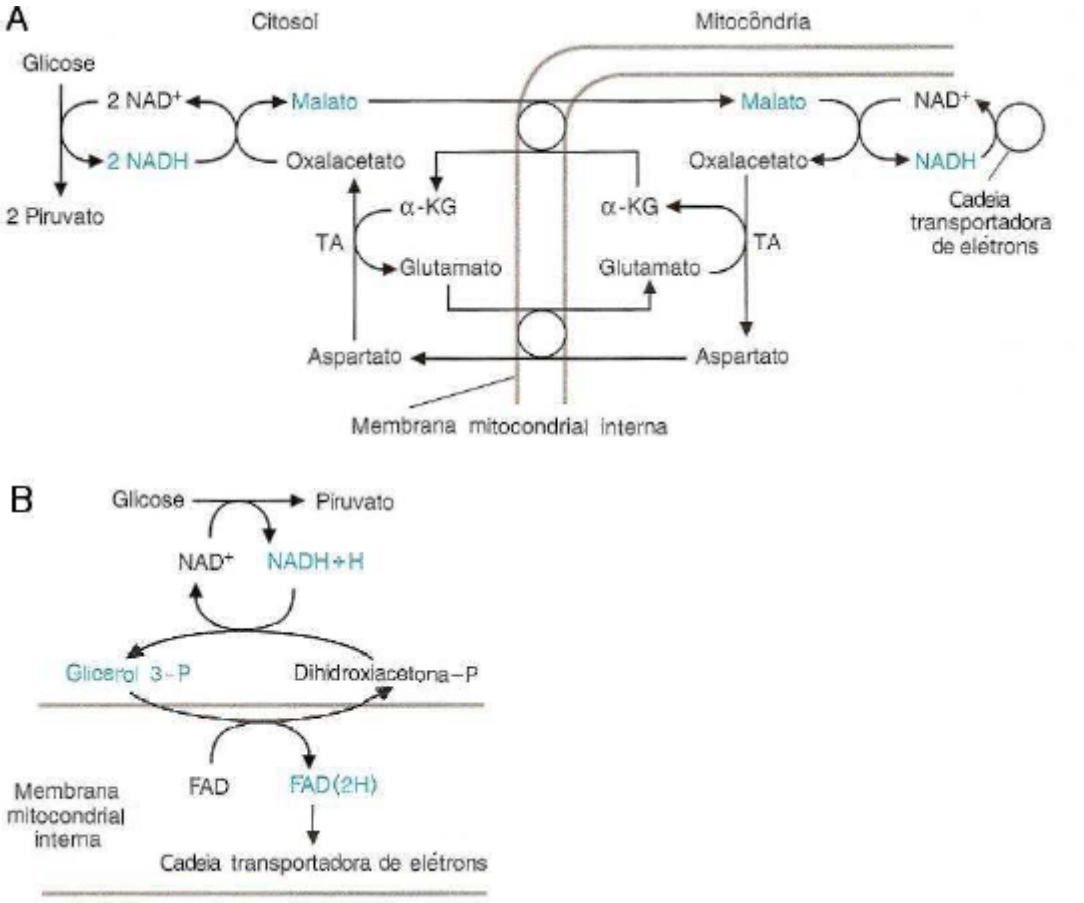

Figura 1. Lançadeira do malato (A) e lançadeira do glicerol-3-P (B) (Adaptado de Smith, Marks e Lieberman, 2005).

Desse modo, a respiração mitocondrial pode ser estimada através da medida do consumo de O_2. Apesar do fato de que essa medida determina diretamente apenas a velocidade de uma única reação (transferência final de elétrons para O_2), muitas informações sobre outros processos mitocondriais podem ser obtidos simplesmente pela adaptação das condições de incubação, tornando o processo de análise significativo na taxa de consumo final. Vários passos podem ser investigados, incluindo o transporte de substratos através da membrana mitocondrial, a atividade das desidrogenases, a atividade dos complexos da cadeia respiratória, o transporte de nucleotídeos de adenina pela

membrana mitocondrial, a atividade da ATP sintase e a permeabilidade da membrana mitocondrial a H^+ (Nicholls e Ferguson, 2002). Experimentalmente, pode-se dividir a respiração mitocondrial em 5 estágios, conforme ilustra a figura 2. No entanto, apenas os parâmetros estados 3 e 4 são comumente utilizados. O estado 3 representa o consumo de oxigênio quando as mitocôndrias, em um meio contendo substrato oxidável, são expostas a ADP, estimulando o consumo de O_2 e produzindo ATP (estado fosforilante). O estado 4 reflete o consumo de O_2 após as mitocôndrias já terem depletado todo o ADP disponível, reduzindo a taxa da respiração (estado não-fosforilante) (Nicholls e Ferguson, 2002). A transdução de energia entre a cadeira respiratória e o gradiente eletroquímico de H^+ é bem regulada, sendo que um pequeno desequilíbrio termodinâmico entre ambos pode resultar em uma alteração importante no transporte de elétrons pela mesma. Assim, quando o gradiente de prótons é dissipado pela ação da ATP sintase, devido à adição de ADP, há um desequilíbrio que estimula a transferência de elétrons pela cadeia respiratória e, consequentemente, o consumo de oxigênio. Sendo assim, para que a ATP sintase esteja ativa, são necessários dois fatores: disponibilidade de ADP e potencial de membrana suficientemente alto (Nelson e Cox, 2000). Nesse contexto, o acoplamento da respiração mitocondrial é definido como a capacidade da mitocôndria gerar energia (ATP) quando exposta ao ADP, ou seja, unir (acoplar) os processos de oxidação e de fosforilação. A dissipação do gradiente eletroquímico de prótons no espaço mitocondrial intermembranas determinado por dano ou aumento da permeabilidade da membrana mitocondrial interna desacopla o transporte de elétrons da síntese de ATP, resultando em um

aumento do consumo de oxigênio (atividade respiratória aumentada) com reduzida formação de ATP (Nicholls e Ferguson, 2002).

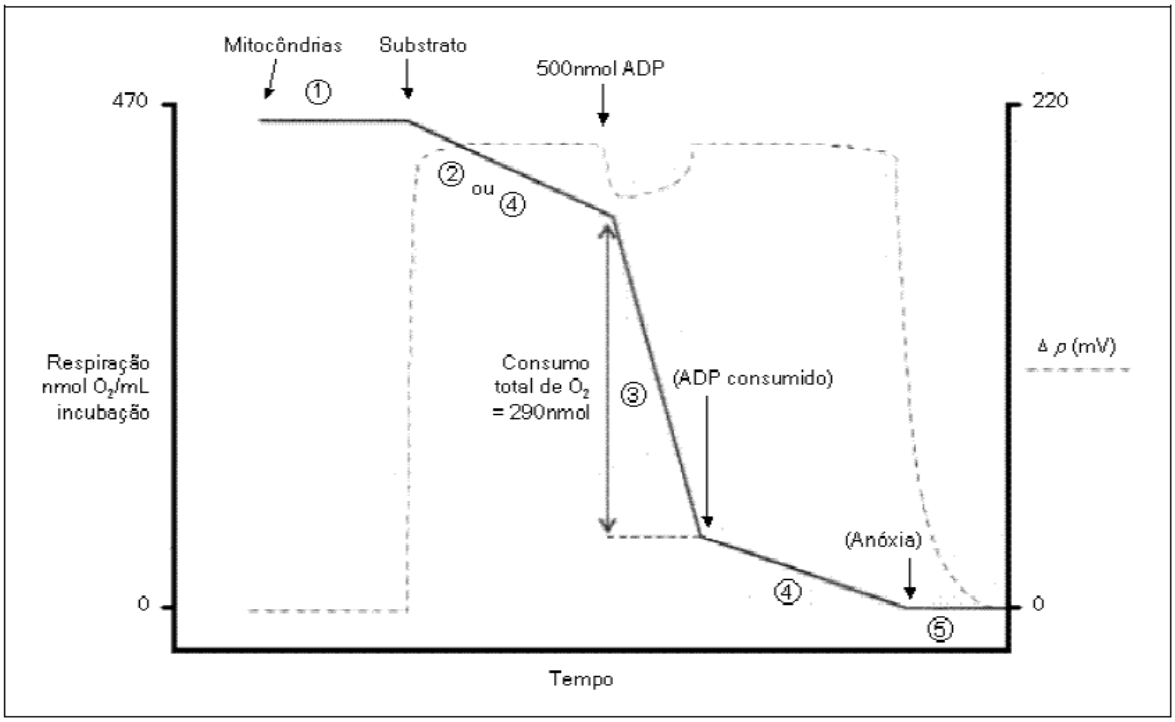

Figura 2. Estados da respiração mitocondrial. (Adaptado de Nicholls e Ferguson, 2002).

Além da regeneração do ATP, que é a sua principal função, a mitocôndria desempenha outros importantes papéis. Esta organela é a principal fonte de espécies reativas de oxigênio e de defesas antioxidantes nas células (Cadenas e Davies, 2000), gerando ânions superóxido ($O_2^{\bullet -}$) no espaço intermembrana pelo vazamento de elétrons que se combinam com o oxigênio molecular no complexo III em um processo que é dependente do potencial de membrana ($\Delta\Psi m$), e na matriz, através de um sítio não definido do complexo I (Han et al., 2001). Além disso, a mitocôndria participa ativamente da homeostase de cálcio (Nicholls e Akerman, 1982) e está envolvida em diversos processos que levam à morte

celular, incluindo liberação de citocromo *c* (Liu et al., 1996). Acredita-se que todos estes processos estejam interligados e que um desequilíbrio nessas funções possa estar envolvido na fisiopatologia de diversas doenças neurodegenerativas, incluindo doenças de Alzheimer, Parkinson, Huntington, esclerose lateral amiotrófica (Beal, 2007; Sasaki et al., 2007; Gil e Rego, 2008; Reddy e Beal, 2008), doenças psiquiátricas, tais como transtorno bipolar, esquizofrenia e depressão (Wang, 2007; Ben-Shacar e Kerry, 2008; Gardner et al., 2008; Shao et al., 2008; Regenold et al., 2009), e também de vários erros inatos do metabolismo (Schuck et al., 2002; Reis de Assis et al., 2004; Latini et al., 2005; Zugno et al., 2006; Ferreira et al., 2007; Mirandola et al., 2008; Moshal et al., 2008; Ribeiro et al., 2008; Viegas et al., 2008).

A função mitocondrial pode ser avaliada pela medida da produção de espécies reativas, do potencial de membrana mitocondrial, do estado redox estimado pelo conteúdo de NAD(P)H/NAD(P)$^+$, e pelo inchamento mitocondrial, que pode ser secundário à abertura do poro de transição mitocondrial.

1.1.6. Radicais Livres

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

Em condições fisiológicas do metabolismo celular aeróbico, o oxigênio molecular (O_2) sofre redução tetravalente, com incorporação de quatro elétrons, resultando na formação de água (H_2O). No entanto, aproximadamente 5% do oxigênio utilizado na cadeia respiratória mitocondrial não são completamente

reduzido à água, podendo ser convertido a intermediários reativos como o radical superóxido ($O_2^{\bullet-}$) e hidroxil ($^{\bullet}OH$), e também a peróxido de hidrogênio (H_2O_2), processo esse que pode ser exacerbado em condições patológicas (Boveris, 1998).

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

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

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

sua função e reagir com o DNA e RNA, levando a mutações somáticas e à distúrbios de transcrição (Delanty e Dichter, 1998; Halliwell e Whiteman, 2004), dentre outros efeitos.

I.1.6.1. Defesas Antioxidantes

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

- agentes que removem cataliticamente os radicais livres, como as enzimas superóxido dismutase, catalase, glutationa peroxidase, entre outras;
- proteínas que minimizam a disponibilidade de pró-oxidantes (íons de ferro e cobre, por exemplo), ao se ligarem aos mesmos como as transferrinas;
- proteínas que protegem biomoléculas de dano oxidativo por outros mecanismos;
- agentes de baixo peso molecular que aprisionam espécies reativas de oxigênio e nitrogênio, como glutationa, α-tocoferol, ácido ascórbico e a bilirrubina.

I.1.6.2. Estresse Oxidativo

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

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

Assim, o termo “estresse oxidativo” é usado para se referir à situação na qual a geração de espécies reativas ultrapassa a capacidade das defesas antioxidantes disponíveis. Pode resultar tanto de uma diminuição das defesas antioxidantes quanto de uma produção aumentada de oxidantes, bem como da liberação de metais de transição ou a combinação de quaisquer desses fatores (Halliwell, 2006).

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

- Adaptação: as células podem tolerar um estresse oxidativo moderado, que geralmente resulta em um aumento da síntese de sistemas de defesa antioxidante a fim de restaurar o balanço oxidante/antioxidante. Apesar disso, nem sempre o estresse oxidativo precisa envolver defesas antioxidantes aumentadas.

- Dano celular: o estresse oxidativo pode danificar alvos moleculares (DNA, proteínas, carboidratos e lipídios) (Halliwell e Gutteridge, 2007). A resposta à injúria pode ser reversível: a célula entra em um estado de homeostase alterado temporário ou prolongado, que não leva à morte celular.

- Morte celular: pode ocorrer tanto por necrose quanto por apoptose. Na morte celular por necrose, a célula incha e se rompe, liberando seu conteúdo para o meio extracelular. Pode haver a liberação de antioxidantes, como a catalase e a glutationa reduzida, e também de pró-oxidantes, como os íons cobre

e ferro e proteínas do grupo heme, agentes esses que podem afetar as células adjacentes, podendo até mesmo induzi-las a um estresse oxidativo. Já na apoptose, o mecanismo de morte celular programada é ativado e não há a liberação do conteúdo celular. A apoptose pode estar acelerada em certas doenças, tais como as desordens neurodegenerativas, havendo envolvimento do estresse oxidativo (Halliwell e Gutteridge, 2007).

I.1.6.3. Estresse Oxidativo e Doenças Neurodegenerativas

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

Numerosas evidências sugerem que os radicais livres e o estresse oxidativo possam estar envolvidos na patogênese do dano neurológico em várias doenças neurodegenerativas. Estudos demonstraram uma diminuição na atividade do complexo I da cadeia respiratória em cérebros *postmortem* de pacientes portadores de doença de Parkinson (Schapira et al., 1990b). Essa inibição do complexo I pode acarretar um aumento na geração de espécies reativas, tais como ânion superóxido, hidroxil e peroxinitrito, as quais poderiam causar um

prejuízo ainda maior na cadeia transportadora de elétrons. Dessa forma, é possível que o estresse oxidativo e a disfunção mitocondrial formem um "ciclo vicioso" na doença de Parkinson (Schapira et al., 1989, 1990a,b; Janetzky et al., 1994; Gu et al., 1998).

Na doença de Alzheimer, a mais comum dentre as doenças neurodegenerativas, é possível que o estresse oxidativo tenha um papel chave na morte neuronal. Tem sido proposto que o peptídeo β-amilóide, o formador das chamadas placas senis, tenha a capacidade de gerar radicais livres espontaneamente. Estudos também evidenciaram um dano oxidativo em cérebros humanos *postmortem* com doença de Alzheimer, através da observação de aumento de 8-hidroxi-2'-deoxiguanosina (8-OHdGA), produtos de oxidação de outras bases e de RNA, carbonilas de proteínas, nitrotirosina e marcadores de peroxidação lipídica (Smith et al., 1991; Markesbery e Carney, 1999; Nourooz-Zadeh et al., 1999; Lovell et al., 2000).

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

Nos últimos anos, foi verificado que vários metabólitos acumulados em alguns EIM induzem estresse oxidativo no cérebro de animais experimentais (Latini et al., 2007; Feksa et al., 2008; Kessler et al., 2008; Leipnitz et al., 2008; Zugno et al., 2008) e em seres humanos (Sitta et al., 2006; Deon et al., 2007;

Ribeiro et al., 2007; Barschak et al., 2008a,b; Deon et al., 2008), indicando que compostos acumulados nestas doenças possam causar dano oxidativo.

I.2. OBJETIVOS

I.2.1. Objetivo Geral

O objetivo geral deste trabalho foi investigar o efeito *in vitro* dos principais metabólitos acumulados na deficiência de MCAD sobre importantes parâmetros de estresse oxidativo e de função mitocondrial em cérebro de ratos jovens, visando a uma melhor compreensão dos mecanismos neurotóxicos desses ácidos graxos.

I.2.2. Objetivos Específicos

- Investigar o efeito *in vitro* dos ácidos octanóico (AO), decanóico (AD) e *cis*-4-decenóico (AcD) sobre o potencial de membrana mitocondrial, sobre o conteúdo dos equivalentes reduzidos de NADH e NADPH e sobre o inchamento mitocondrial em preparações mitocondriais de cérebro de ratos;
- Investigar o efeito *in vitro* do ácido *cis*-4-decenóico (AcD) sobre a liberação de peróxido de hidrogênio em preparações mitocondriais de cérebro de ratos;
- Investigar o efeito *in vitro* dos ácidos octanóico (AO), decanóico (AD) e *cis*-4-decenóico (AcD) sobre os parâmetros respiratórios estados 3 e 4, índice de controle respiratório (RCR) e a razão ADP/O, medidos através do consumo de oxigênio em preparações mitocondriais de cérebro de ratos;
- Investigar o efeito *in vitro* dos ácidos octanóico (AO) e decanóico (AD) sobre as atividades dos complexos da cadeia transportadora de elétrons I-IV em preparações mitocondriais de cérebro de ratos;

- Investigar o efeito *in vitro* dos ácidos octanóico (AO), decanóico (AD) e *cis*-4-decenóico (AcD) sobre dano oxidativo lipídico (quimioluminescência espontânea e níveis de substâncias reativas ao ácido tiobarbitúrico), dano oxidativo protéico (formação de carbonilas e o conteúdo de grupamentos tióis) e as defesas antioxidantes não-enzimáticas (potencial antioxidante total do tecido – TRAP e concentração de glutationa reduzida) em homogeneizado de córtex cerebral de ratos de 30 dias de vida;
- Investigar o efeito *in vitro* do ácido *cis*-4-decenóico (AcD) sobre a formação de espécies ativas (ânion superóxido e oxidação da 2',7'-di-hidrodiclorofluoresceína – DCFH) e sobre a atividade das enzimas antioxidantes catalase, superóxido dismutase e glutationa peroxidase em córtex cerebral de ratos de 30 dias de idade.

PARTE II

Artigos Científicos

Capítulo I

cis-4-Decenoic acid promotes mitochondrial dysfunction in rat brain

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Moacir Wajner

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Journal of Inherited Metabolic Disease

cis-4-Decenoic acid promotes mitochondrial dysfunction in rat brain

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Summary

High tissue levels of *cis*-4-decenoic acid (cDA) are pathognomonic of medium chain acyl-CoA dehydrogenase (MCAD) deficiency. Affected patients present encephalopathic crises characterized by seizures, lethargy that may lead to coma and death, whose pathophysiology is unknown. In the present work, we investigated the *in vitro* effect of cDA on oxygen consumption, mitochondrial membrane potential, H_2O_2 release, NAD(P)H content and swelling in mitochondrial preparations from rat brain, using glutamate plus malate or succinate as substrates. cDA increased state 4 and decreased state 3 respiration and the respiratory control and ADP/O ratios. cDA also markedly reduced the mitochondrial membrane potential, H_2O_2 production and NAD(P)H content and provoked mitochondrial swelling. Moreover, the increased rate of oxygen consumption was not prevented by atractyloside, indicating that cDA effect did not involve the adenine nucleotide transporter. Similarly, mitochondrial swelling occurred despite the use of cyclosporin A (CsA), ruling out a role for the mitochondrial permeabilization transition pore (MPTP). These data strongly suggest that cDA leads to impairment of mitochondrial homeostasis acting as an uncoupler of oxidative phosphorylation, probably due a non-selective permeabilization of the inner mitochondrial membrane through other mechanisms than CsA-sensitive MPTP opening. It is therefore presumed that bioenergetic impairment provoked by cDA may be involved in the brain dysfunction observed in MCAD-deficient patients.

Synopsis: *cis*-4-cecenoic acid promotes mitochondrial dysfunction in rat brain

Abbreviated Title: *cis*-4-cecenoic acid and mitochondrial dysfunction

List of Abbreviations: ATC, atractyloside; BSA, bovine serum albumine; CCCP, carbonyl cyanide m-chloro phenyl hydrazone; cDA, *cis*-4-decenoic acid; CsA, cyclosporin A; DA, decanoic acid; DTNB, 5',5''-dithiobis-(2-nitrobenzoate); EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; FET, forward electron transfer; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]; MCAD, medium-chain acyl-CoA dehydrogenase; MCADD, medium-chain acyl-CoA dehydrogenase deficiency; MCFA, medium-chain fatty acid; MOPS, 3-[N-morpholino]propanesulfonic acid; OA, octanoic acid; RCR, respiratory control ratio; RET, reverse electron transfer; $\Delta\Psi m$, mitochondrial membrane potential.

Introduction

Individuals affected by medium-chain acyl-CoA dehydrogenase (MCAD; E.C. 1.3.99.3) deficiency (MCADD), the most common inherited defect of fatty acid oxidation, present severe episodes of hypoketotic hypoglycemia and encephalopathy with seizures, lethargy that may lead to coma and sudden death (Onkenhout et al 1995; Rinaldo et al 1998; Roe and Ding 2001). During these crises, which are generally precipitated by prolonged fasting or infection, the levels of the accumulating metabolites are dramatically increased (Derks et al 2006; Mayell et al 2007). *cis*-4-Decenoic (cDA) accumulates in tissue and body fluids of patients affected by MCADD and is considered pathognomonic of this disease. Clinical management relies on the administration of high glucose and L-carnitine amounts during the acute episodes, as well as fat restriction, fasting avoidance and L-carnitine supplementation after recovery (Coates 1994; Roe and Ding 2001).

In spite of the high prevalence of this disorder in the general population, little is known about the pathomechanisms responsible for the neurologic symptoms in MCADD. Hypoglycemia may acutely affect the central nervous system. However, in many cases the encephalopathic crises occur in the absence of low blood glucose levels, suggesting that the accumulating compounds may be neurotoxic. In this context, there are only a few studies describing the deleterious effects of cDA, as well as of octanoic acid (OA) and decanoic acid (DA), which also accumulate in this disorder. Thus, *in vitro* studies demonstrated that OA, DA and cDA impair several parameters of energy metabolism including activities of the respiratory chain, mitochondrial creatine kinase and Na^+,K^+-ATPase in cerebral cortex of rats (de Assis et al 2003, 2006; Reis

de Assis et al 2004), with cDA eliciting the most pronounced effects. In addition, cDA was recently demonstrated to provoke oxidative stress in the brain of young rats (Schuck et al 2007). Based on these findings, we hypothesized that cDA may be the most toxic metabolite in MCADD. Considering that *trans*,*trans*-2,4-decadienal, that is a compound structurally similar to cDA, strongly impair mitochondrial homeostasis promoting non-selective inner mitochondrial membrane permeabilization (Sigolo et al 2008), in the present work we investigated the *in vitro* effect of cDA on various mitochondrial parameters, including oxygen consumption, mitochondrial membrane potential ($\Delta\Psi m$), hydrogen peroxide (H_2O_2) production, NAD(P)H content and mitochondrial swelling in rat brain mitochondrial preparations.

Material and Methods

Reagents

All chemicals were purchased from Sigma (St. Louis, MO, USA), except for *cis*-4-decenoic acid (cDA) which was prepared by Dr. Ernesto Brunet, Madrid, Spain with 99 % purity. cDA was dissolved on the day of the experiments in the incubation medium used for each technique with pH adjusted to 7.4. The final concentrations of the acids in the medium ranged from 0.1 to 1.0 mM.

Animals

Thirty-day-old Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS and Biotério do Conjunto das Químicas, USP were used.

Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22±1°C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul and Universidade de São Paulo. All efforts were made to minimize the number of animals used and their suffering.

Preparation of mitochondrial fractions

Forebrain mitochondria were isolated from 30-day-old rats as described by Rosenthal et al (1987), with slight modifications. Animals were sacrificed by decapitation, had their brains rapidly removed and put into ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (BSA; free fatty acid), 10 mM HEPES pH 7.2 and 5 mg protease. The cerebellum and underlying structures were removed and the remaining material was used as the forebrain. The tissue was cut into small pieces using surgical scissors and extensively washed. The tissue was then manually homogenized in a Dounce homogenizer using both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2000 x *g*. After centrifugation, the supernatant was again centrifuged for 8 min at 12000 x *g*. The pellet was resuspended in 20 mL of isolation buffer containing 10 uL of 10% digitonin and recentrifuged for 8 min at 12000 x *g*. The supernatant was discarded and the final pellet gently washed and resuspended in isolation buffer devoid of EGTA, at an approximate protein concentration

of 25-35 mg . mL^{-1}. This preparation results in a mixture of synaptosomal and non-synaptosomal mitochondria similar to the general brain composition.

Citrate Synthase Activity

All the experiments described here were carried out after checking the integrity of the inner mitochondrial membrane by measuring the activity of citrate synthase (CS) in the absence and in the presence of Triton X-100. CS activity was measured according to Srere (1969) in a medium containing 0.1 mM 5',5''-dithiobis-(2-nitrobenzoate) (DTNB), 0.2 mM oxaloacetic acid, 0.1 % Triton X-100, 0.1 mM acetyl-CoA and 100 mM Tris-HCl, pH 8.0. Only mitochondria with a CS latency of 95% or more were used for the experiments

Oxygen Consumption

The rate of oxygen consumption and the ADP/O ratio were measured polarographically using a Clark-type electrode in a thermostatically controled (37ºC) and magnetically stirred incubation chamber. cDA (0.1 – 1.0 mM) was added to the reaction medium at the beginning of the assay. The assay was performed in purified mitochondrial preparations (0.75 mg . mL^{-1} when 2.5 mM malate plus 2.5 mM glutamate were used as substrates, and 0.50 mg . mL^{-1} when succinate was used as substrate) incubated in a buffer containing 0.3 M sucrose, 5 mM MOPS, 5 mM potassium phosphate, 1 mM EGTA and 0.1% BSA. State 3 mitochondrial respiration was measured after addition of 1 mM ADP to the incubation medium. In order to measure state 4 mitochondrial respiration, 1 μg . mL^{-1} oligomycin A was added to the incubation medium. The respiratory control ratio (RCR;

state 3/state 4) was then calculated. States 3 and 4 were expressed as nmol O_2 consumed . min^{-1} . mg of $protein^{-1}$. The ADP/O ratio was estimated according to Estabrook (1967), using 100 μM ADP in the incubation medium. Only mitochondrial preparations with RCR higher than 4 were used in the experiments.

Measurement of Mitochondrial Inner Membrane Potentials (ΔΨm)

Mitochondrial inner membrane potentials (ΔΨm) were estimated according to Akerman and Wikström (1976) and Kowaltowski et al (2002) by following the fluorescence of the 5 μM cationic dye safranin O at an excitation wavelength of 495 nm and emission wavelength of 586 nm, using 5 nm slits, on a Hitachi F-4500 spectrofluorometer, using 2.5 mM malate plus 2.5 mM glutamate or 5 mM succinate as substrates, in the presence of 1 μg . mL^{-1} oligomycin A. Data were expressed as fluorescence arbitrary units (FAU).

Measurement of Hydrogen Peroxide (H_2O_2) Release

Hydrogen peroxide production was evaluated according to Mohanty et al (1997), by measuring the oxidation of 50 μM Amplex Red (Molecular Probes) in the presence of 1.0 units. mL^{-1} horseradish peroxidase. The incubation medium contained 0.6 M sorbitol, 20 mM Tris-HCl buffer pH 7.5, and 0.5 mM EDTA, 5 mM succinate as substrate. The rate of Amplex Red oxidation was recorded at 37ºC using a Hitachi F-4500 spectrofluorometer operating at excitation and emission wavelengths of 563 and 587 nm, respectively. H_2O_2 release was expressed as nmol H_2O_2 . min^{-1} . mg of $protein^{-1}$.

Determination of NAD(P)H Fluorescence

Matrix NAD(P)H autofluorescence was measured in an incubation medium containing 0.6 M sorbitol, 20 mM Tris-HCl buffer pH 7.5, and 0.5 mM EDTA, using 2.5 mM malate plus 2.5 mM glutamate as substrates. Mitochondria (0.5 mg . mL^{-1}) were incubated at 37ºC in the assay medium and the fluorescence was measured using 366 nm excitation and 450 nm emission wavelenghts. Data were expressed as nmol NAD(P)H . mg of $protein^{-1}$.

Mitochondrial Swelling

Mitochondrial swelling was followed by measuring light scattering changes on a temperature-controlled Hitachi F-4500 spectrofluorometer with magnetic stirring operating at excitation and emission of 520 nm, using succinate as substrate. Data were expressed as fluorescence arbitrary units (FAU).

Protein Determination

Protein was measured by the method of Lowry et al (1967) using bovine serum albumin as standard.

Statistical Analysis

Results are presented as mean ± standard deviation. Assays were performed in duplicate and the mean was used for statistical analysis. Data was analyzed using one-way analysis of

variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. For analysis of dose-dependent effects and correlations tests, linear regression was used. Only significant F values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out using the Statistical Package for the Social Sciences (SPSS) software.

Results

This investigation used brain mitochondrial preparations to uncover possible changes in cerebral bioenergetic function provoked by cDA. Although results obtained from purified mitochondria may differ from *in vivo* effects, the experimental setting allows for ready quantifications of bioenergetic parameters and manipulations of experimental conditions not possible in whole brain.

We first determined the respiratory parameters in the absence or presence of cDA (Figure 1). It can be observed in the figure that rat brain mitochondria incubated under our conditions were fully coupled, as indicated by the difference in respiratory rates observed in the presence of ADP (state 3) and after the addition of the ADP synthase inhibitor oligomycin (state 4). Interestingly, the addition of cDA increased oxygen consumption rates in state 4 respiration regardless of the substrate used [glutamate/malate: $F(3,15) = 25.2$; $P < 0.001$; succinate: $F(3,15) = 8.69$; $P < 0.01$]. However, this fatty acid only diminished state 3 respiration (up to 68%) when succinate was used as substrate [$F(3,15) = 7.80$; $P < 0.01$]. cDA was also able to decrease the ADP/O ratio [glutamate/malate: $F(3,15) = 18.8$; $P < 0.001$; succinate: $F(3,15) = 30.1$; $P < 0.001$] and the respiratory control ratio (RCR) [glutamate/malate: $F(3,15) = 19.5$; $P < 0.001$; succinate: $F(3,15) = 45.1$; $P < 0.001$]

regardless of the substrate, suggesting that this compound leads to uncoupling of mitochondrial respiration from ATP synthesis.

In order to confirm that cDA promotes uncoupling, mitochondrial membrane potentials were measured using the fluorescent probe safranin O (Figure 2). We observed that cDA markedly decreased ΔΨm with either glutamate plus malate or succinate as substrates. Furthermore, the addition of the protonophore CCCP was unable to change the ΔΨm decrease induced by cDA, reinforcing a relevant uncoupling effect of this fatty acid.

Considering that uncouplers modulate H_2O_2 levels, we evaluated the role of cDA on mitochondrial H_2O_2 generation (Figure 3). We observed that cDA, at concentrations ranging from 0.1 mM to 1 mM, significantly reduced H_2O_2 release [$F(7,22) = 29.6$; $P < 0.001$]. It was also verified that cDA provoked a higher effect than that of rotenone, which decreases H_2O_2 formation by inhibiting the reverse electron transport (RET), whereas the co-incubation of cDA and rotenone resulted in an effect of the same magnitude to that caused by cDA alone. These data indicate that, besides inhibiting the RET, cDA effect may be also due to other mechanisms than uncoupling and RET inhibition.

Uncoupling effects are generally accompanied by reduction of the mitochondrial matrix NAD(P)H pool. Therefore, we assayed the NAD(P)H content. We observed that cDA provoked a significant decrease in NAD(P)H fluorescence (Figure 4). Furthermore, at 1.0 mM cDA there was an immediate drop in the NAD(P)H autofluorescence. Similar results were obtained when CCCP was added to the incubation medium (data not shown). Furthermore, the supplementation of rotenone to the medium did not fully reestablish the amount of reduced equivalents, when compared to controls, indicating that NAD(P)H pool was partly lost from the matrix.

In order to gain insight into the mechanism through which cDA uncouples mitochondria, we assessed oxygen consumption in the presence of 1 μM atractyloside (ATC), an inhibitor of adenine nucleotide transporter, which is able to prevent FA-induced uncoupling effect in mitochondrial preparations. We found that the effect of cDA increasing oxygen consumption was not prevented by ATC [$F(3,15) = 27.3$; $P < 0.001$] (Figure 5), ruling out a selective mitochondrial membrane permeabilization via the adenine nucleotide transporter in the cDA-elicited uncoupling effect.

The next step of our investigation was to study whether the cDA-elicited ΔΨm decrease was related to non-selective inner membrane permeabilization, we measured the mitochondrial swelling following light scattering of the mitochondrial suspension. We observed that cDA provoked an extensive mitochondrial swelling (Figure 6). However, the addition of cyclosporin A (CsA) was not able to prevent the cDA-elicited mitochondrial swelling, excluding a role for classical mitochondrial permeability transition (MTP) in this effect.

Discussion

Patients affected by MCADD present encephalopathic crises accompanied by cerebral abnormalities (Smith and Davis 1973; Ruitenbeek et al 1995; Mayatepek et al 1997; Wilson et al 1999; Mayell et al 2007), whose pathogenesis is not yet defined. Lethargy that may progress to coma and death is believed to be particularly due to the accumulation of toxic medium-chain fatty acids (MCFA) and their by-products (Gregersen et al 2008). It may be therefore presumed that the predominant metabolites that accumulate

in this disorder, namely octanoate (OA), decanoate (DA) and *cis*-4-decenoate (cDA), contribute to the neurological symptoms of the affected patients.

Previous data reported that cDA, the pathognomonic compound accumulating in MCADD, impairs brain mitochondrial bioenergetics to a higher extend than OA and DA, and also causes oxidative damage (Reis de Assis et al 2004; Schuck et al 2007). These data suggest that cDA may be the most toxic compound in MCADD. cDA is structurally similar compound to *trans*,*trans*-2,4-decadienal, a promoter of non-selective inner mitochondrial membrane permeabilization (Sigolo et al 2008). Therefore, in the present work we investigated the effects of cDA on various mitochondrial parameters reflecting uncoupling of oxidative phosphorylation, such as oxygen consumption, mitochondrial membrane potential ($\Delta\Psi m$), H_2O_2 production, NAD(P)H content and mitochondrial swelling in rat brain mitochondrial preparations in the hope to unravel some mechanisms involved in MCADD pathophysiology.

We first found that cDA, at micromollar concentrations, significantly increased state 4 (non-ADP-stimulated mitochondria) respiration rate with succinate or glutamate plus malate. Interestingly, when succinate was used as substrate, the rate of oxygen consumption was lower at a high cDA dose (1.0 mM), which could be due to a lower complex II activity, limiting the flux of electrons by the respiratory chain, as previously demonstrated (Reis de Assis et al 2004). cDA was also able to markedly inhibit ADP-stimulated respiration rate in mitochondria supported by succinate (state 3), with no effect when glutamate plus malate were used as substrates. In addition, cDA provoked a significant decrease of RCR and ADP/O ratios in mitochondria supported by all substrates tested (glutamate plus malate and succinate). Taken together, these observations indicate that cDA impairs the efficiency of oxidative phosphorylation, acting as an uncoupler and as a metabolic inhibitor.

Likewise, our findings showing that cDA also provoked a decrease of ΔΨm in non-ADP-stimulated mitochondria (state 4) with a similar effect to that of CCCP further supports an uncoupling role for cDA.

We also observed that cDA was able to impair H_2O_2 production in rat brain mitochondria when succinate was used as substrate. This is in line with the increased oxygen consumption rate and decreased ΔΨm caused by this fatty acid. On the other hand, inhibition of complex II activity and/or of the reverse electron transfer (RET) could also explain these findings. It should be stressed that H_2O_2 is formed as a side-product of the respiratory chain and can be released by succinate-supported mitochondria through two distinct pathways: RET and forward electron transfer (FET). RET occurs when electrons driven from complex II reduce NAD^+ to NADH in complex I passing through CoQ, a process which occurs in the presence of high ΔΨm (Nicholls and Ferguson 2002). FET is the pathway by which electrons are transferred to complex IV leading to O_2 reduction to H_2O (Nicholls and Ferguson 2002).

We searched for the mechanism by which cDA reduced H_2O_2 release, by adding rotenone (Rot), at a concentration that totally prevents RET, into succinate respiring mitochondria. We observed that rotenone did not modify the cDA-induced decrease of H_2O_2 release, implying a role for RET in this cDA effect. In addition, it was found that rotenone *per se* decreased H_2O_2 release to a lesser degree than cDA, suggesting that other mechanisms are involved in cDA action on H_2O_2 release. Possible explanations for these findings could be an uncoupling effect, inhibition of the electron transfer and NAD(P)H loss that could affect H_2O_2 release.

We also found that cDA significantly altered the mitochondrial redox state by reducing the matrix NAD(P)H content, which is commonly caused by uncouplers.

However, rotenone did not totally reverse the decrease of NADH and NADPH levels caused by cDA, implying that the reduced equivalents may have been partially lost from the mitochondrial matrix.

Involvement of the adenine nucleotide transporter (ANT) in the uncoupling effect elicited by fatty acids has been previously demonstrated (Brustovetskyl et al 1990; Skulachev 1998; Samartsev et al 2000). Therefore, we assessed the mitochondrial oxygen consumption in the presence of ATC, an inhibitor of this transporter. The rate of oxygen consumption was not altered by ATC, suggesting that cDA probably acts through a distinct mechanism than that used by other fatty acids. In this scenario, cDA could interact with phospholipid from the mitochondrial membrane bilayer, leading to an alteration in fluidity and ion permeability (Kimmelberg and Pahadjopoulos 1974; Lee 1976; Abeywardena et al 1983). Alternatively, cDA could also interfere with anion transporters (Skulachev 1999; Mokhova and Khailova 2005).

Further experiments showing that cDA provoked a marked mitochondrial swelling that was not prevented by cyclosporin A (CsA) probably rules out an effect of this fatty acid on the mitochondrial permeability transition pore (MPTP). Taken together the data on mitochondrial swelling and NAD(P)H decrease, it may be presumed that cDA provokes a non-selective permeabilization of the inner mitochondrial membrane through other mechanisms than CSA-sensitive MPTP opening.

We cannot establish at present the exact pathophysiological significance of our data. However, although the brain cDA concentrations in MCADD are still unknown, our findings demonstrate an impairment of mitochondrial homeostasis (rate of oxygen consumption, RCR and ADP/O ratio, membrane potential, H_2O_2 release, NAD(P)H levels and mitochondrial swelling) at cDA concentrations similar to those found in plasma of

MCADD affected patients (Duran et al 1988). It should be also stressed that during metabolic crises the concentrations of the accumulating metabolites dramatically increase in these patients (Martínez et al 1997; Roe and Ding 2001). Furthermore, it has been previously postulated that the concentrations of organic acids in neural cells overcome those of plasma and of CSF in various organic acidemias (Hoffmann et al 1993). This may be the case for MCADD, since *in vivo* OA administration alters organic acid transport in rat choroid plexus, leading to impairment of the transchoroidal clearance of OA and similar compounds (Kim et al 1983). This effect was suggested to contribute to the accumulation of medium-chain fatty acids in the brain and cerebral spinal fluid of patients affected by Reye syndrome, a disorder in which OA also accumulates. Furthermore, considering that the enzymes of fatty acid oxidation, including MCAD, are expressed in the neural cells (Tyni et al 2004), it is presumed that accumulation of the MCFA occur in the brain.

In conclusion, to our knowledge this is the first report showing that cDA provokes mitochondrial dysfunction in the brain that could be involved in the cerebral abnormalities and neurological symptoms found in MCADD patients (Egidio et al 1989; Maegawa et al 2008). It was shown that cDA impairs the efficiency of oxidative phosphorylation, acting as an uncoupler, through a distinct mechanism than that found for other fatty acids, and also as a metabolic inhibitor of succinate oxidation. The present insights should further stimulate the research to better understand the involvement of mitochondrial dysfunction in the detrimental chain of events leading to neuronal degeneration (disease development) and possibly exploit potential mitochondrial targets for therapeutic interventions in MCADD.

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References

Abeywardena MY, Allen TM, Charnock JS (1983) Lipid-protein interactions of reconstituted membrane-associated adenosine triphosphatases. *Biochim Biophys Acta* 729: 62–74.

Akerman KE, Wikström MK (1976) Safranine as a probe of the mitochondrial membrane potential. *FEBS Lett* 68: 191-197.

Brustovetskyl NN, Dedukhova VI, Egoroval MV, Mokhova EN, Skulachev VP (1990) Inhibitors of the ATP/ADP antiporter suppress stimulation of mitochondrial respiration and H^+ permeability by palmitate and anionic detergents. *FEBS Lett* 272: 187-189.

Coates PM (1994) New developments in the diagnosis and investigation of mitochondrial fatty acid oxidation disorders. *Eur J Pediatr* 153: 49-56.

de Assis DR, Maria RC, Ferreira GC, Schuck PF, Latini A, Dutra-Filho CS, Wannmacher CM, Wyse AT, Wajner M (2006) Na^+, K^+ ATPase activity is markedly reduced by *cis*-4-decenoic acid in synaptic plasma membranes from cerebral cortex of rats. *Exp Neurol* 197: 143-149.

de Assis DR, Ribeiro CA, Rosa RB, Schuck PF, Dalcin KB, Vargas CR, Wannmacher CM, Dutra-Filho CS, Wyse AT, Briones P, Wajner M (2003) Evidence that antioxidants prevent the inhibition of Na^+,K^+-ATPase activity induced by octanoic acid in rat cerebral cortex *in vitro*. *Neurochem Res* 28: 1255-1263.

Derks TG, Reijngoud DJ, Waterham HR, Gerver WJ, van den Berg MP, Sauer PJ, Smit GP (2006) The natural history of medium-chain acyl CoA dehydrogenase deficiency in the Netherlands: clinical presentation and outcome. *J Pediatr* 148: 665-670.

Duran M, Bruinvis L, Ketting D, de Klerk JB, Wadman SK (1988) *Cis*-4-decenoic acid in plasma: a characteristic metabolite in medium-chain acyl-CoA dehydrogenase deficiency. *Clin Chem* 34: 548-551.

Egidio RJ, Francis GL, Coates PM, Hale DE, Roesel A (1989) Medium-chain acyl-CoA dehydrogenase deficiency. *Am Fam Physician* 39:221-226.

Estabrook RW (1967) Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. In Estabrook RW, Pullman ME, eds. *Methods in Enzymology*. Academic Press, New York, 41–47.

Gregersen N, Andresen BS, Pedersen CB, Olsen RK, Corydon TJ, Bross P (2008) Mitochondrial fatty acid oxidation defects-remaining challenges. *J Inherit Metab Dis* 31: 643-657.

Hoffmann GF, Seppel CK, Holmes B, Mitchell L, Christen HJ, Hanefeld F, Rating D, Nyhan WL (1993) Quantitative organic acid analysis in cerebrospinal fluid and plasma: reference values in a pediatric population. *J Chromatogr* 617: 1-10.

Kim CS, O'tuama LA, Mann JD, Roe CR (1983) Effect of increasing carbon chain length on organic acid transport by the choroid plexus: a potential factor in Reye's syndrome. *Brain Res* 259: 340–343.

Kimmelberg H, Pahadjopoulos D (1974) Effects of phospholipid acyl chain fluidity, phase transitions, and cholesterol on (Na^+,K^+)-stimulated adenosine triphosphatase. *J Biol Chem* 249: 1071–1080.

Kowaltowski AJ, Cosso RG, Campos CB, Fiskum G (2002) Effect of Bcl-2 overexpression on mitochondrial structure and function. *J Biol Chem* 277: 42802-42807.

Lee AG (1976) Model for action of local anesthetics. *Nature* 262: 545–548.

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

Maegawa GH, Poplawski NK, Andresen BS, Olpin SE, Nie G, Clarke JT, Teshima I (2008) Interstitial deletion of 1p22.2p31.1 and medium-chain acyl-CoA dehydrogenase deficiency in a patient with global developmental delay. *Am J Med Genet* 146:1581-1586.

Martínez G, Jiménez-Sánchez G, Divry P, Vianey-Saban C, Riudor E, Rodés M, Briones P, Ribes A (1997) Plasma free fatty acids in mitochondrial fatty acid oxidation defects. *Clin Chim Acta* 267: 143-154.

Mayatepek E, Koch HG, Hoffmann GF (1997) Hyperuricaemia and medium-chain acyl-CoA dehydrogenase deficiency. *J Inherit Metab Dis* 20: 842-843.

Mayell SJ, Edwards L, Reynolds FE, Chakrapani AB (2007) Late presentation of medium-chain acyl-CoA dehydrogenase deficiency. J Inherit Metab Dis 30: 104.

Mohanty JG, Jaffe JS, Schulman ES, Raible DG (1997) A highly sensitive fluorescent micro-assay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative. *J Immunol Methods* 202: 133-141.

Mokhova EN, Khailova LS (2005) Involvement of mitochondrial inner membrane anion carriers in the uncoupling effect of fatty acids. *Biochemistry (Mosc)* 70: 159-163.

Nicholls DG, Ferguson SJ (2002) The Chemiostatic proton circuit. In Nicholls DG, Ferguson SJ, eds. *Bioenergetics 3*. Academic Press, London, 57-87.

Onkenhout W, Venizelos V, van der Poel PFH, Van der Heuvel MPM, Poorthuis BJHM (1995) Identification and quantification of intermediates of unsaturated fatty acid metabolism in plasma of patients with fatty acid oxidation disorders. *Clin Chem* 41: 1467–1474.

Reis de Assis D, Maria RC, Borba Rosa R, Schuck PF, Ribeiro CA, da Costa Ferreira G, Dutra-Filho CS, de Souza Wyse AT, Duval Wannmacher CM, Santos Perry ML, Wajner M (2004) Inhibition of energy metabolism in cerebral cortex of young rats by the medium-chain fatty acids accumulating in MCAD deficiency. *Brain Res* 1030: 141-151.

Rinaldo P, Raymond K, Al-Odaib A, Bennett M (1998) Clinical and biochemical features of fatty acid oxidation disorders. *Curr Opin Pediatr* 10: 615–621.

Roe CR, Ding J (2001) Mitochondrial fatty acid oxidation disorders. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Bases of Inherited Disease*. New York: McGraw-Hill, New York , 1909–1963.

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

Ruitenbeek W, Poels PJ, Turnbull DM, Garavaglia B, Chalmers RA, Taylor RW, Gabreels FJ (1995) Rhabdomyolysis and acute encephalopathy in late onset medium chain acyl-CoA dehydrogenase deficiency. *J Neurol Neurosurg Psychiatry* 58: 209-214.

Samartsev VN, Simonyan RA, Markova OV, Mokhova EN, Skulachev VP (2000) Comparative study on uncoupling effects of laurate and lauryl sulfate on rat liver and skeletal muscle mitochondria. *Biochim Biophys Acta* 145: 179-190.

Schuck PF, Ceolato PC, Ferreira GC, Tonin A, Leipnitz G, Dutra-Filho CS, Latini A, Wajner M. (2007) Oxidative stress induction by *cis*-4-decenoic acid: relevance for MCAD deficiency. *Free Radic Res* 41: 1261-1272.

Sigolo CA, Di Mascio P, Kowaltowski AJ, Garcia CC, Medeiros MH (2008) *trans*,*trans*-2,4-decadienal induces mitochondrial dysfunction and oxidative stress. *J Bioenerg Biomembr* 40: 103-109.

Skulachev VP (1998) Uncoupling: new approaches to an old problem of bioenergetics. *Biochim Biophys Acta* 1363: 100-124.

Skulachev VP (1999) Anion carriers in fatty acid-mediated physiological uncoupling. *J Bioenerg Biomembr* 31: 431-445.

Smith Jr ET, Davis GJ (1993) Medium-chain acylcoenzyme-A dehydrogenase deficiency. Not just another Reye syndrome. *Am J Forensic Med Pathol* 14: 313-318.

Srere PA (1969) Citrate Synthase. *Methods Enzymol* 13: 3-11.

Tyni T, Paetau A, Strauss AW, Middleton B, Kivelä T (2004) Mitochondrial fatty acid beta-oxidation in the human eye and brain: implications for the retinopathy of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency. *Pediatr Res* 56: 744-750.

Wilson CJ, Champion MP, Collins JE, Clayton PT, Leonard JV (1999) Outcome of medium chain acyl-CoA dehydrogenase defciency after diagnosis. *Arch Dis Child* 80: 459–462.

Figures Legends

Figure 1. Effect of cDA on oxygen consumption in ADP-stimulated (state 3) and non-ADP-stimulated (state 4) mitochondria supported by glutamate/malate *(A)* or succinate *(B)*, on respiratory control ratio (RCR) *(C)* and on ADP/O ratio *(D)*. After addition of mitochondria (0.5 mg . mL^{-1}), different concentrations of cDA (0.1-1.0 mM) were added to the incubation medium. Values are means ± standard deviation for four to five independent experiments and states 3 and 4 of mitochondrial respiration are expressed as nmol O_2. min^{-1}. mg of $protein^{-1}$. **P*<0.05 ***P*<0.01, ****P*<0.001 compared to controls (Duncan multiple range test).

Figure 2. Effect of cDA on mitochondrial membrane potential using glutamate/malate *(A)* or succinate *(B)* as substrates. After addition of mitochondria (0.5 mg . mL^{-1}), cDA was given in 0.1-1.0 mM concentrations. CCCP (1 μM) was added at the end of the measurements. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU).

Figure 3. Effect of cDA on H_2O_2 generation in mitochondria supported by succinate. After addition of mitochondria (0.5 mg . mL^{-1}), different concentrations of cDA (0.1-1.0 mM) were added to the incubation medium. Rotenone (Rot) was used at a final concentration of 4 μM. Values are means ± standard deviation for four independent experiments and are expressed as nmol H_2O_2. min^{-1}. mg $protein^{-1}$. ***P*<0.01, ****P*<0.001 compared to controls (Duncan multiple range test).

Figure 4. Effect of cDA on mitochondrial NAD(P)H content using glutamate/malate *(A)* or succinate *(B)* as substrates. After addition of mitochondria (0.5 mg . mL^{-1}), cDA was given in 1.0 mM concentration. Rotenone (4 μM) was added at the end of the measurements. Data were expressed as nmol NAD(P)H . mg of $protein^{-1}$. Traces are representative of four independent experiments.

Figure 5. Effect of cDA on oxygen consumption in glutamate/malate-supported mitochondria respiring in state 4. After addition of mitochondria (0.5 mg . mL^{-1}), 1 mM cDA was added to the incubation medium in the presence or in the absence of 1 μM atractyloside (ATC). Values are means ± standard deviation for four independent experiments and state 4 of mitochondrial respiration is expressed as nmol O_2. min^{-1}. mg of $protein^{-1}$. ***P<0.001 compared to controls (Duncan multiple range test).

Figure 6. Effect of cDA on mitochondrial swelling. After addition of mitochondria (0.5 mg . mL^{-1}), cDA was given in 1.0 mM concentration. Cyclosporyn A (CsA) was used at a final concentration of 1 μM. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU).

Figure 1.

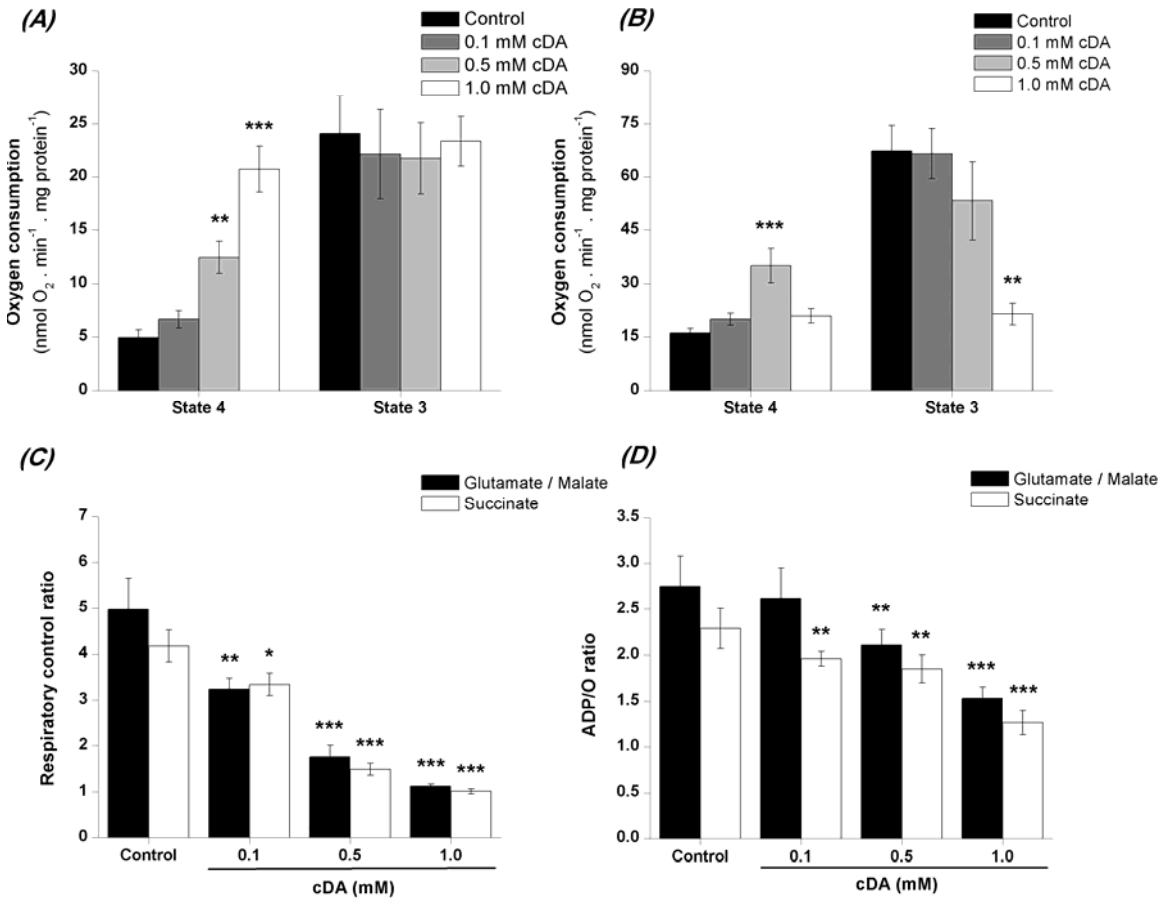

(A)
Control
0.1 mM cDA
0.5 mM cDA
1.0 mM cDA
Oxygen consumption
(nmol O_2 . min^{-1} . mg $protein^{-1}$)
State 4
State 3
(B)
(C)
Glutamate / Malate
Succinate
Respiratory control ratio
Control
0.1
0.5
1.0
cDA (mM)
(D)
ADP/O ratio

Figure 2.

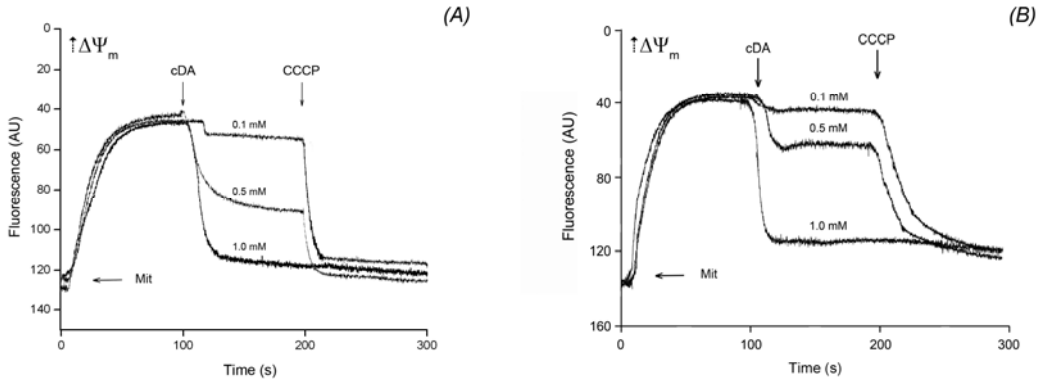

(A)
↑ΔΨm
cDA
CCCP
0.1 mM
0.5 mM
1.0 mM
Mit
Fluorescence (AU)
Time (s)
(B)
↑ΔΨm
cDA
CCCP
0.1 mM
0.5 mM
1.0 mM
Mit
Fluorescence (AU)
Time (s)

Figure 3.

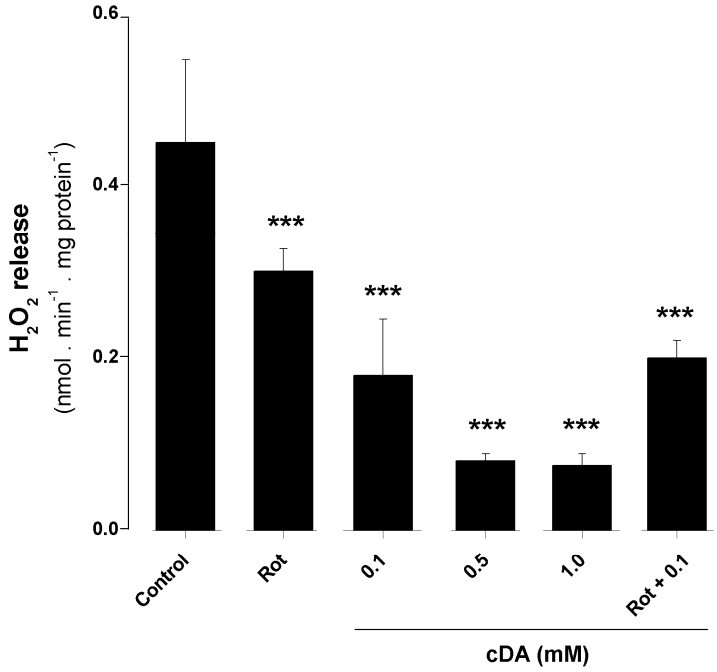

0.6
0.4
0.2
0.0
H_2O_2 release
(nmol . min^{-1} . mg $protein^{-1}$)
***
***
***
***
***
Control
Rot
0.1
0.5
1.0
Rot + 0.1
cDA (mM)

Figure 4.

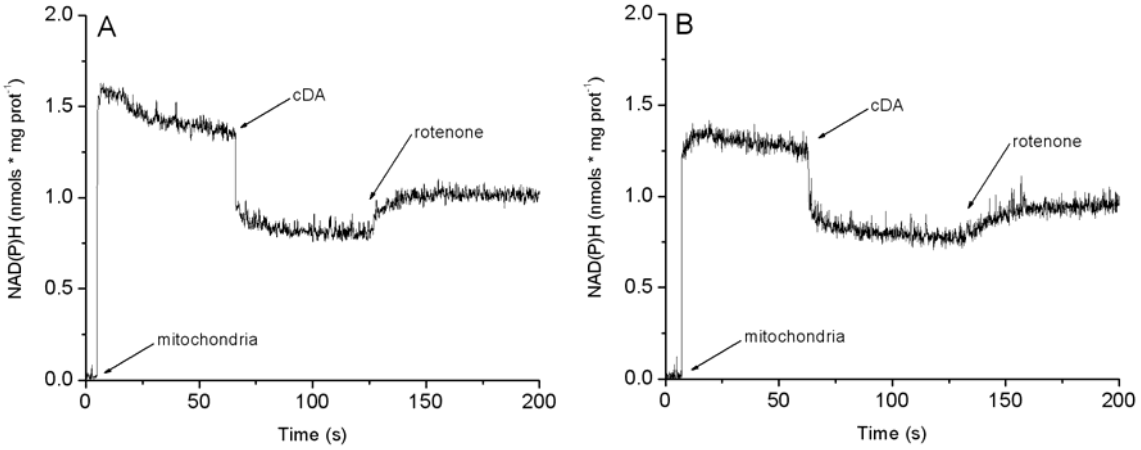

A
B
cDA
rotenone
mitochondria
NAD(P)H (nmols * mg prot^{-1})
Time (s)
0.0
0.5
1.0
1.5
2.0
0
50
100
150
200

Figure 5.

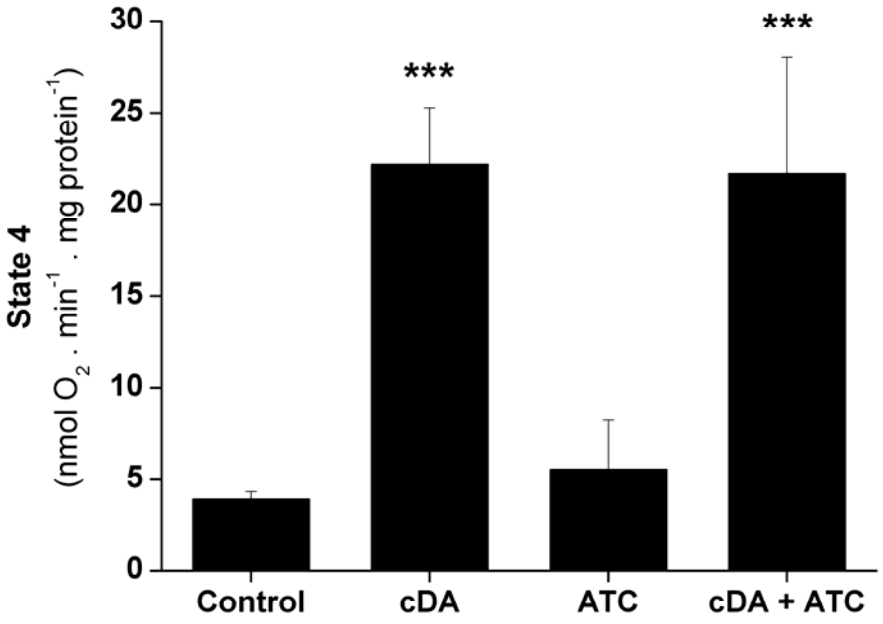
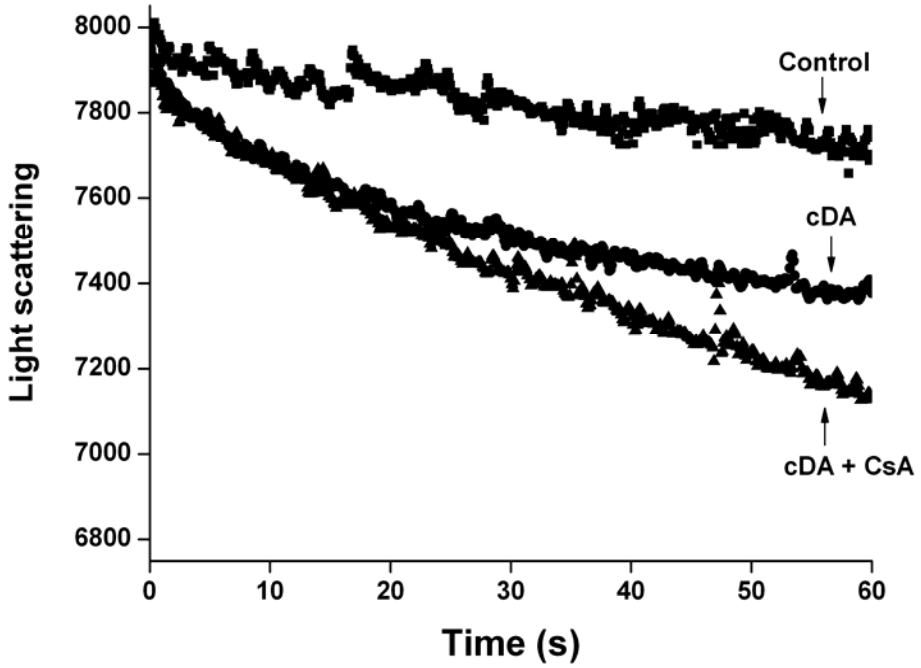

State 4
(nmol O_2 . min^{-1} . mg $protein^{-1}$)
30
25
20
15
10
5
0
***
***
Control
cDA
ATC
cDA + ATC

Figure 6.


8000
7800
7600
7400
7200
7000
6800
Light scattering
0
10
20
30
40
50
60
Time (s)
Control
cDA
cDA + CsA

Capítulo II

Evidence that the major metabolites accumulating in MCAD deficiency disturb mitochondrial homeostasis in rat brain

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Brain Research

Evidence that the major metabolites accumulating in MCAD deficiency disturb mitochondrial homeostasis in rat brain

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Abstract

Medium-chain acyl-CoA dehydrogenase deficiency (MCADD) is an inherited metabolic disorder of fatty acid oxidation in which the affected patients present predominantly high levels of octanoic (OA) and decanoic (DA) acids in tissues and body fluids. It is clinically characterized by encephalopathic crises, as well as by progressive neurological deterioration, whose pathophysiology is poorly known. In the present work, we investigated the *in vitro* effects of OA and DA on various parameters of mitochondrial homeostasis in mitochondrial preparations from rat brain. We found that OA and DA markedly increased state 4 respiration and diminished state 3 respiration, the respiratory control and ADP/O ratios and mitochondrial membrane potential, suggesting that these fatty acids may act as uncouplers of oxidative phosphorylation. OA and DA also inhibited state U respiration and the respiratory chain complex activities. Furthermore, the uncoupler effect of DA was partially prevented by atractyloside, indicating an involvement of the adenine nucleotide transporter in the DA-elicited uncoupling effect. OA and DA also reduced the matrix NAD(P)H levels. Finally, DA, but not OA, provoked a marked mitochondrial swelling, reflecting non-selective permeabilization of the inner mitochondrial membrane caused by DA. Taken together, these data suggest that OA and DA impair mitochondrial homeostasis in the brain and this may possibly represent a pathomechanism involved in the neurological abnormalities present in MCADD.

Section: Disease Related Neuroscience

Keywords: octanoic acid, decanoic acid, MCAD deficiency, oxidative phosphorylation, brain, rat

Introduction

Octanoic (OA) and decanoic (DA) acids are the predominant metabolites accumulating in medium chain acyl-CoA dehydrogenase (MCAD; E.C. 1.3.99.3) deficiency (MCADD), the most common inherited defect of fatty acid oxidation (Okenhunt et al., 1995; Rinaldo et al., 1998; Roe and Ding, 2001). Glycine and L-carnitine bind to these organic acids giving rise to derivatives that also accumulate in this disorder (Okenhunt et al., 1995). The clinical presentation typically occurs in early childhood, but can occasionally be delayed until adulthood (Ruitenbeek et al. 1995; Raymond et al., 1999; Feillet et al., 2003). The major features of the disease include hypoglycemia, vomiting, lethargy and encephalopathy after fasting, infection or other metabolic stressors, such as surgery. Management of the acute illness includes rapid correction of hypoglycemia, rehydration and treatment of the underlying infection or other stress factor. Current long-term therapy includes avoidance of fasting and a high carbohydrate, low-fat diet (Roe and Ding, 2001), but it does not fully prevent the crises and neurodegeneration (Wilcken et al., 1994, 2007).

In spite of the high prevalence of this disorder in the general population, few studies have been carried out to investigate the deleterious effects elicited by MCAD accumulating metabolites on cell homeostasis. In this context, it has been demonstrated that OA and DA disturb energy metabolism in rat brain, reducing the efficiency of glycolytic and TCA cycle rates, inhibiting some activities of the respiratory chain complexes and creatine kynase in the brain and increasing oxygen consumption in the liver (Scholz et al., 1984; Reis de Assis et al., 2004).

In the present work, we studied the effect of OA and DA on important parameters of mitochondrial bioenergetics, including the respiratory parameters evaluated by oxygen consumption, mitochondrial membrane potential ($\Delta\Psi m$), the activities of respiratory chain complexes I-IV, NAD(P)H levels and mitochondrial swelling in brain mitochondrial preparations from young rats in order to better characterize the deleterious influence of these fatty acids on mitochondria homeostasis.

Results

This study used mitochondrial preparations from rat brain to identify possible alterations of bioenergetic function provoked by OA or DA. Even though the results obtained using purified mitochondria may differ from *in vivo* effects, this approach allows for ready quantifications of bioenergetic parameters and manipulations of experimental conditions not possible in whole brain.

We first determined the influence of OA and DA on respiratory parameters by measuring the rate of oxygen consumption (Figures 1 and 2, respectively). It can be observed in the figures that rat brain mitochondria incubated under our conditions were fully coupled, as indicated by the difference in respiratory rates observed in the presence of ADP (state 3) and after the addition of the ADP synthase inhibitor oligomycin A (state 4). We found that both fatty acids increased state 4 respiration regardless of the substrate used [OA: glutamate/malate: $F(3,15) = 9.38$; $P < 0.001$; succinate: $F(3,15) = 15.8$; $P < 0.001$; DA: glutamate/malate: $F(3,15) = 49.4$; $P < 0.001$; succinate: $F(3,15) = 18.6$; $P < 0.001$]. DA was also able to decrease the respiratory control ratio (RCR) [glutamate/malate: $F(3,15) = 30.2$; $P < 0.001$; succinate: $F(3,15) = 36.1$; $P < 0.001$] and the ADP/O ratio [glutamate/malate: $t(5) = 3.19$; $P < 0.05$; succinate: $F(3,15) = 13.7$; $P < 0.001$] with both

substrates. In addition, OA diminished the RCR [glutamate/malate: $F(3,15) = 7.55$; $P < 0.01$; succinate: $F(3,15) = 15.4$; $P < 0.001$] but did not alter the ADP/O ratio. These results suggest that OA and DA may behave as uncouplers of the oxidative phosphorylation.

Interestingly, the rate of oxygen consumption was lower at 3.0 mM as compared to 1.0 mM DA in state 4 respiration, suggesting that this fatty acid provoked a metabolic inhibition at high concentrations. In addition, state 3 respiration was decreased by DA in the presence of both substrates [glutamate/malate: $F(3,15) = 50.6$; $P < 0.001$; succinate: $F(3,15) = 46.6$; $P < 0.001$], but only in the presence of succinate by OA [$F(3,15) = 5.62$; $P < 0.01$]. We then investigated whether the decrease in the rate of oxygen consumption in state 4 respiration caused by high concentrations of OA and DA could be due to alterations of the transport of substrates into the mitochondrial matrix, mitochondrial dehydrogenase activities or electron transfer through the respiratory chain. We then assessed the rate of oxygen consumption using uncoupled mitochondria in the presence of CCCP (state U) in order to search for a distinct mechanism than uncoupling for OA and DA effects. We found that both fatty acids reduced this parameter, with DA presenting a more pronounced effect [$F(2,11) = 44.8$; $P < 0.001$] (Figure 3), indicating that the oxidative system is impaired by OA and DA. We also verified that complexes I-III and IV activities were inhibited by both fatty acids [I-III: $F(2,11) = 12.5$; $P < 0.01$; IV: $F(2,11) = 34.1$; $P < 0.001$], whereas complex II-III activity was only affected by DA [$F(2,11) = 8.56$; $P < 0.01$] (Figure 4). The latter results indicated that mitochondrial oxidative system is affected by high concentrations of OA and DA by reducing the electron flow through the respiratory chain.

To further evaluate the uncoupling effect of OA and DA, we determined whether OA and DA could alter the mitochondrial membrane potential ($\Delta\Psi m$) using the fluorescent probe safranin O (Figure 5). Both fatty acids significantly decreased $\Delta\Psi m$ in state 4-

respiring mitochondria using glutamate plus malate or succinate as substrates, corroborating an uncoupling effect promoted by these metabolites. However, DA was more effective decreasing ΔΨm, as compared to OA. Furthermore, the addition of the protonophore CCCP was not able to change the ΔΨm decrease induced by DA, suggesting that maximal uncoupling was already achieved by the presence of this fatty acid.

In order to gain insight into the mechanism through which OA and DA uncouple mitochondria, we assessed oxygen consumption in state 4 respiration in the presence of 1 μM atractyloside (ATC), an inhibitor of adenine nucleotide transporter (Figure 6). We found that ATC was able to prevent the effect of DA increasing oxygen consumption in state 4 respiring mitochondria [$F(5,23) = 9.92$; $P < 0.001$], but not that of OA. These results indicate that the adenine nucleotide transporter is likely to be involved in the DA-elicited uncoupling effect.

Since uncoupling effects are generally accompanied by reduction of the mitochondrial matrix NAD(P)H pool, we therefore assayed the matrix NAD(P)H content (Figure 7). We observed that both fatty acids decreased the matrix NAD(P)H fluorescence. Furthermore, at 3.0 mM of OA or DA, we observed an immediate drop in the NAD(P)H autofluorescence, resembling the results obtained when CCCP was added to the incubation medium (data not shown). Further addition of rotenone to the medium fully reverted the OA-elicited decrease in reduced equivalents pool, but only partially the DA-elicited effect, indicating that NAD(P)H pool was partly lost from the matrix when DA was present in the incubation medium.

The final step of our investigation was to investigate whether the ΔΨm decrease elicited by OA and DA could be related to non-selective inner membrane permeabilization

by measuring the mitochondrial swelling following light scattering of the mitochondrial suspensions (Figure 8). We observed that DA caused an extensive mitochondrial swelling, with no effect for OA, suggesting that DA probably promotes a non-selective permeabilization of the inner mitochondrial membrane.

Discussion

The mechanisms underlying the neurologic damage in patients affected by medium-chain acyl-CoA dehydrogenase deficiency (MCADD) are so far poorly understood. However, toxicity of the fatty acids and their by-products accumulating in this disorder has recently been hypothesized as involved in its pathophysiology (Gregersen et al., 2008). Therefore, it may be presumed that the increased brain concentrations of octanoate (OA) and decanoate (DA), the major metabolites that are accumulated in MCADD, contribute to the neurological symptoms of the affected patients.

In this context, previous studies demonstrated that medium-chain fatty acid (MCFA), which dramatically increase during crisis of metabolic decompensation in MCADD, inhibit respiratory chain complex activities in rat brain homogenates and impair oxygen consumption altering mitochondrial respiratory parameters in rat liver (Scholz et al., 1984; Reis de Assis et al., 2004). In the present work we investigated the effect of OA and DA on crucial parameters of cerebral bioenergetics by using mitochondrial preparations obtained from brain of young rats. The parameters analysed were respiratory parameters assessed by oxygen consumption, the activities of respiratory chain complexes I-IV, mitochondrial membrane potential ($\Delta\Psi m$), NAD(P)H content and mitochondrial swelling.

We first observed that OA and DA, even at micromollar concentrations, significantly increased state 4 (non-ADP-stimulated mitochondria) respiration supported by glutamate plus malate or succinate. In addition, state 3 respiration was decreased by OA in the presence of succinate as substrate and by DA in the presence of both substrates. Moreover, these fatty acids provoked a significant decrease of RCR and DA also diminished ADP/O ratio in mitochondria supported by all tested substrates (glutamate plus malate and succinate). Taken together, these observations indicate that OA and DA act as uncouplers of oxidative phosphorylation. However, the rate of oxygen consumption in state 4 respiration was lower at the highest tested DA dose (3.0 mM) when compared to 1 mM, indicating that, besides uncoupling, other mechanisms involving metabolic inhibition may be acting in DA-elicited effect. It is conceivable that an impairment of the oxidative system, which includes transport of substrates into the mitochondrial matrix, matrix dehydrogenases and electron transfer through the respiratory chain, could explain the decrease in the rate of oxygen consumption in state 4 respiration caused by the DA at high concentrations. We therefore assessed the uncoupled state (state U) of the mitochondrial respiration, by adding CCCP, which allow us to investigate the oxidative system with no interference of the uncoupling effect. We found that both OA and mainly DA decreased state U, implying that other mechanisms besides uncoupling are implicated in the actions induced by these MCFA. We then searched for inhibitions on the respiratory chain provoked by OA and DA and observed that both fatty acids inhibited complexes I-III and IV of the respiratory chain, and DA also inhibited complex II-III. Thus, the effects of OA and DA affecting the respiratory rate in ADP-stimulated and non-ADP-stimulated mitochondria may be at least partially explained by inhibition of electron transfer through respiratory chain. However, we cannot rule out that OA and DA provoke alteration of the

transport of substrates (glutamate/aspartate exchanger and succinate transporter), mitochondrial matrix dehydrogenase enzyme activities, or even cytochrome *c* release, which may induce similar findings.

Our next findings showing that OA and especially DA provoked a significant decrease of ΔΨm in state 4-respiring mitochondria with a comparable effect to CCCP further support an uncoupling role for these fatty acids.

Possible involvement of the adenine nucleotide transporter (ANT) in the uncoupling effect elicited by fatty acids has been previously demonstrated (Brustovetskyl et al., 1990; Skulachev, 1998; Samartsev et al., 2000). Therefore, we assessed the rate of oxygen consumption in state 4- respiring mitochondria in the presence of ATC, an inhibitor of this transporter. Although ATC was not able to alter the effect of OA increasing the rate of oxygen consumption, it did attenuate DA-elicited effect, implying that DA probably acts through a mechanism involving the ANT. Other mechanisms that could be involved in the uncoupling effect promoted by these fatty acids include interactions with phospholipids from the mitochondrial membrane bilayer, leading to an alteration in fluidity and ion permeability (Kimmelberg and Pahadjopoulos, 1974; Lee, 1976; Abeywardena et al., 1983). We cannot exclude that OA and DA could also interfere with anion transporters (Skulachev, 1999; Mokhova and Khailova, 2005).

OA and DA also significantly reduced the matrix NAD(P)H levels, a finding commonly provoked by uncouplers. Moreover, rotenone was able to fully reestablish reduced equivalents levels with OA but only partially when DA was present in the incubation medium, implying that the reduced equivalents may have been partially lost from the mitochondrial matrix in the presence of DA. In this context, DA, but not OA, provoked a marked mitochondrial swelling, which possibly reflects a greater membrane

permeability induced by this fatty acid. It may be concluded that a non-selective permeabilization of the inner mitochondrial membrane caused by DA could allow a partial loss of the reduced equivalents from the mitochondrial matrix.

At present, we cannot establish the exact pathophysiological significance of our data. However, even though the brain OA and DA concentrations in MCADD are not established yet, it should be stressed that OA and mainly DA provoked an impairment of mitochondrial homeostasis at concentrations similar to those found in plasma of MCADD affected patients (Duran et al., 1988; Onkenhout et al., 1995). It should be also mentioned that during metabolic crises the concentrations of the accumulating metabolites dramatically increase in these patients (Martínez et al., 1997; Roe and Ding, 2001). Furthermore, it has been previously postulated that the concentrations of organic acids in neural cells overcome those of plasma of CSF in various organic acidemias (Hoffmann et al., 1993). This may be the case for MCADD, since *in vivo* OA administration alters organic acid transport in rat choroid plexus, leading to impairment of the transchoroidal clearance of OA and similar compounds (Kim et al., 1983). This effect was suggested to contribute to the accumulation of medium chain fatty acids in the brain and cerebral spinal fluid of patients affected by Reye syndrome, a disorder in which OA also accumulates. Furthermore, considering that the enzymes of fatty acid oxidation, including MCAD, are expressed in the neural cells (Tyni et al., 2004), it is presumed that accumulation of the MCFA occur in the brain.

In conclusion, we showed in the present work that OA and DA, the major metabolites accumulating in MCADD, provoke mitochondrial dysfunction, by acting as uncouplers and metabolic inhibitors of the oxidative phosphorylation. We also found that these fatty acids impairing mitochondrial respiration through distinct mechanisms. The

uncoupling effect of DA was shown to involve the ANT, distinctly from OA. Furthermore, DA induced a severe mitochondrial swelling, which may potentially trigger a chain of events resulting in cell death (Green and Reed, 1998), whereas OA did not elicit this effect. It is therefore presumed that the these effects, allied to previous results from the literature (Reis de Assis et al., 2004), could explain at least in part the cerebral abnormalities and neurological symptoms found in MCADD patients (Egidio et al., 1989; Maegawa et al., 2008).

Experimental Procedure

Reagents

All chemicals were purchased from Sigma (St. Louis, MO, USA). OA and DA were dissolved on the day of the experiments in the incubation medium used for each technique with pH adjusted to 7.4. The final concentrations of these fatty acids in the medium ranged from 0.5 to 3.0 mM.

Animals

Thirty-day-old Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS were used. Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22±1°C) colony room. The “Principles of Laboratory Animal Care”

(NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul. All efforts were made to minimize the number of animals used and their suffering.

Preparation of mitochondrial fractions

Forebrain mitochondria were isolated from 30-day-old rats as described (Rosenthal et al., 1987), with slight modifications. Animals were sacrificed by decapitation, had their brains rapidly removed and put into ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (BSA; free fatty acid), 10 mM HEPES pH 7.2 and 5 mg protease. The cerebellum and underlying structures were removed and the remaining material was used as the forebrain. The tissue was cut into small pieces using surgical scissors and extensively washed. The tissue was then manually homogenized in a Dounce homogenizer using both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2000 x *g*. After centrifugation, the supernatant was again centrifuged for 8 min at 12000 x *g*. The pellet was resuspended in 20 mL of isolation buffer containing 10 uL of 10% digitonin and recentrifuged for 8 min at 12000 x *g*. The supernatant was discarded and the final pellet gently washed and resuspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 25-35 mg . mL^{-1}. This preparation results in a mixture of synaptosomal and non-synaptosomal mitochondria similar to the general brain composition.

Oxygen Consumption

The rate of oxygen consumption and the ADP/O ratio were measured polarographically using a Clark-type electrode in a thermostatically controled (37ºC) and magnetically stirred incubation chamber. OA and DA (0.5 – 3.0 mM) were added to the reaction medium at the beginning of the assay. The assay was performed in purified mitochondrial preparations (0.75 mg . mL^{-1} when 2.5 mM malate plus 2.5 mM glutamate were used as substrates, and 0.50 mg . mL^{-1} when succinate was used as substrate) incubated in a buffer containing 0.3 M sucrose, 5 mM MOPS, 5 mM potassium phosphate, 1 mM EGTA and 0.1% BSA. State 3 mitochondrial respiration was measured after addition of 1 mM ADP to the incubation medium. In order to measure state 4 mitochondrial respiration, 1 μg . mL^{-1} oligomycin A was added to the incubation medium. The respiratory control ratio (RCR; state 3/state 4) was then calculated. We also measured the rate of oxygen consumption on uncoupled mitochondrial preparations (state U) by adding the proton ionophore CCCP (1 μM) to the incubation medium. States 3, 4 and U were expressed as nmol O_2 consumed . min^{-1} . mg of $protein^{-1}$. The ADP/O ratio was estimated according to Estabrook (1967), using 100 μM ADP in the incubation medium. Only mitochondrial preparations with RCR higher than 5 were used in the experiments.

Spectrophotometric analysis of the respiratory chain complexes I–IV activities

The activities of the respiratory chain complexes I-III, II-III and IV were determined in mitochondrial preparations from rat brain, according to standard methods slightly modified, as described previously (da C Ferreira et al., 2005). The enzyme activities were calculated as nmol min^{-1} mg $protein^{-1}$.

Measurement of Mitochondrial Inner Membrane Potentials (ΔΨm)

Mitochondrial inner membrane potentials (ΔΨm) were estimated according to Akerman and Wikström (1976) and Kowaltowski et al., 2002 by following the fluorescence of the 5 μM cationic dye safranin O at an excitation wavelength of 495 nm and emission wavelength of 586 nm, using 5 nm slits, on a Hitachi F-4500 spectrofluorometer, using 2.5 mM malate plus 2.5 mM glutamate or 5 mM succinate as substrates, in the presence of 1 μg . mL^{-1} oligomycin A. Data were expressed as fluorescence arbitrary units (FAU).

Determination of NAD(P)H Fluorescence

Matrix NAD(P)H autofluorescence was measured in an incubation medium containing 0.6 M sorbitol, 20 mM Tris-HCl buffer pH 7.5, and 0.5 mM EDTA, using 2.5 mM malate plus 2.5 mM glutamate as substrates. Mitochondria (0.5 mg . mL^{-1}) were incubated at 37ºC in the assay medium and the fluorescence was measured using 366 nm excitation and 450 nm emission wavelenghts. Data were expressed as nmol NAD(P)H . mg of $protein^{-1}$.

Mitochondrial Swelling

Mitochondrial swelling was followed by measuring light scattering changes on a temperature-controlled Hitachi F-4500 spectrofluorometer with magnetic stirring operating at excitation and emission of 520 nm, using succinate as substrate. Data were expressed as fluorescence arbitrary units (FAU).

Protein Determination

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

Statistical Analysis

Results were presented as mean ± standard deviation. Assays were performed in duplicate and the mean was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. For analysis of dose-dependent effects, linear regression was used. Only significant F values were shown in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out using the Statistical Package for the Social Sciences (SPSS) software.

Acknowledgements

This work was supported by grants from CNPq, PRONEX II, FAPERGS, PROPESQ/UFRGS, FAPESP and FINEP research grant Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00.

Literature References

Abeywardena, M.Y., Allen, T.M., Charnock, J.S., 1983. Lipid-protein interactions of reconstituted membrane-associated adenosine triphosphatases. Biochim. Biophys. Acta 729, 62–74.

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

Brustovetskyl, N.N., Dedukhova, V.I., Egoroval, M.V., Mokhova, E.N., Skulachev, V.P., 1990. Inhibitors of the ATP/ADP antiporter suppress stimulation of mitochondrial respiration and H^+ permeability by palmitate and anionic detergents. FEBS Lett. 272, 187-189.

da C Ferreira, G., Viegas, C.M., Schuck, P.F., Latini, A., Dutra-Filho, C.S., Wyse, A.T., Wannmacher, C.M., Vargas, C.R., Wajner, M., 2005. Glutaric acid moderately compromises energy metabolism in rat brain. Int. J. Dev. Neurosci. 23**,** 687-693.

Duran, M., Bruinvis, L., Ketting, D., de Klerk, J.B., Wadman, S.K., 1988. *Cis*-4-decenoic acid in plasma: a characteristic metabolite in medium-chain acyl-CoA dehydrogenase deficiency. Clin. Chem. 34, 548-551.

Egidio, R.J., Francis, G.L., Coates, P.M., Hale, D.E., Roesel, A., 1989. Medium-chain acyl-CoA dehydrogenase deficiency. Am. Fam. Physician. 39, 221-226.

Estabrook, R.W., 1967. Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. In Methods in Enzymology, R.W. Estabrook, M.E. Pullman, ed. Academic Press, New York, pp 41–47.

Feillet, F., Steinmann, G., Vianey-Saban, C., de Chillou, C., Sadoul, N., Lefebvre, E., Vidailhet, M., Bollaert, P.E., 2003. Adult presentation of MCAD deficiency revealed by coma and severe arrhythmias. Intensive Care Med. 29, 1594 –1597.

Green, D.R., Reed, J.C., 1998. Mitochondria and apoptosis. Science. 281, 1309-1312.

Gregersen, N., Andresen, B.S., Pedersen, C.B., Olsen, R.K., Corydon, T.J., Bross, P., 2008. Mitochondrial fatty acid oxidation defects-remaining challenges. J. Inherit. Metab. Dis. 31**,** 643-657.

Hoffmann, G.F., Seppel, C.K., Holmes, B., Mitchell, L., Christen, H.J., Hanefeld, F., Rating, D., Nyhan, W.L., 1993. Quantitative organic acid analysis in cerebrospinal fluid and plasma: reference values in a pediatric population. J. Chromatogr. 617, 1-10.

Kim, C.S., O'tuama, L.A., Mann, J.D., Roe, C.R., 1983. Effect of increasing carbon chain length on organic acid transport by the choroid plexus: a potential factor in Reye's syndrome. Brain Res. 259, 340–343.

Kimmelberg, H., Pahadjopoulos, D., 1974. Effects of phospholipid acyl chain fluidity, phase transitions, and cholesterol on (Na^+,K^+)-stimulated adenosine triphosphatase. J. Biol. Chem. 249, 1071–1080.

Kowaltowski, A.J., Cosso, R.G., Campos, C.B., Fiskum, G., 2002. Effect of Bcl-2 overexpression on mitochondrial structure and function. J. Biol. Chem. 277, 42802-42807.

Lee, A.G., 1976. Model for action of local anesthetics. Nature 262, 545–548.

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

Maegawa, G.H., Poplawski, N.K., Andresen, B.S., Olpin, S.E., Nie, G., Clarke, J.T., Teshima, I., 2008. Interstitial deletion of 1p22.2p31.1 and medium-chain acyl-CoA dehydrogenase deficiency in a patient with global developmental delay. Am. J. Med. Genet. 146, 1581-1586.

Martínez, G., Jiménez-Sánchez, G., Divry, P., Vianey-Saban, C., Riudor, E., Rodés, M., Briones, P., Ribes, A., 1997. Plasma free fatty acids in mitochondrial fatty acid oxidation defects. Clin. Chim. Acta 267, 143-154.

Mokhova, E.N., Khailova, L.S., 2005. Involvement of mitochondrial inner membrane anion carriers in the uncoupling effect of fatty acids. Biochemistry (Mosc.) 70, 159-163.

Onkenhout, W., Venizelos, V., van der Poel, P.F.H., Van der Heuvel, M.P.M., Poorthuis, B.J.H.M., 1995. Identification and quantification of intermediates of unsaturated fatty acid metabolism in plasma of patients with fatty acid oxidation disorders. Clin. Chem. 41, 1467–1474.

Raymond, K., Bale, A.E., Barnes, C.A., Rinaldo, P., 1999. Medium-chain acyl Co-A dehydrogenase deficiency: sudden and unexpected death of a 45 year old woman. Genet. Med. 1, 293– 294.

Reis de Assis, D., Maria, R.C., Borba Rosa, R., Schuck, P.F., Ribeiro, C.A., da Costa Ferreira, G., Dutra-Filho, C.S., Terezinha de Souza Wyse, A., Duval Wannmacher, C.M., Santos Perry, M.L., Wajner. M., 2004. Inhibition of energy metabolism in cerebral cortex

of young rats by the medium-chain fatty acids accumulating in MCAD deficiency. Brain Res. 1030, 141-151.

Rinaldo, P., Raymond, K., Al-Odaib, A., Bennett, M., 1998. Clinical and biochemical features of fatty acid oxidation disorders. Curr. Opin. Pediatr. 10, 615–621.

Roe, C.R., Ding, J., 2001. Mitochondrial fatty acid oxidation disorders. In *The* Metabolic and Molecular Bases of Inherited Disease, C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, ed. New York: McGraw-Hill, New York , pp 1909–1963.

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

Ruitenbeek, W., Poels, P.J., Turnbull, D.M., Garavaglia, B., Chalmers, R.A., Taylor, R.W., Gabreels, F.J., 1995. Rhabdomyolysis and acute encephalopathy in late onset medium chain acyl-CoA dehydrogenase deficiency. J. Neurol. Neurosurg. Psychiatry 58, 209-214.

Samartsev, V.N., Simonyan, R.A., Markova, O.V., Mokhova, E.N., Skulachev, V.P., 2000. Comparative study on uncoupling effects of laurate and lauryl sulfate on rat liver and skeletal muscle mitochondria. Biochim. Biophys. Acta 145, 179-190.

Scholz, R., Schwabe, U., Soboll, S., 1984. Influence of fatty acids on energy metabolism. 1. Stimulation of oxygen consumption, ketogenesis and CO_2 production following addition of octanoate and oleate in perfused rat liver. Eur. J. Biochem. 141**,** 223-230.

Skulachev, V.P., 1998. Uncoupling: new approaches to an old problem of bioenergetics. Biochim. Biophys. Acta 1363, 100-124.

Skulachev, V.P., 1999. Anion carriers in fatty acid-mediated physiological uncoupling. J. Bioenerg. Biomembr. 31, 431-445.

Tyni, T., Paetau, A., Strauss, A.W., Middleton, B., Kivelä, T., 2004. Mitochondrial fatty acid beta-oxidation in the human eye and brain: implications for the retinopathy of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency. Pediatr. Res. 56, 744-750.

Wilcken, B., Haas, M., Joy, P., Wiley, V., Chaplin, M., Black, C., Fletcher, J., McGill, J., Boneh, A., 2007. Outcome of neonatal screening for medium-chain acyl-CoA dehydrogenase deficiency in Australia: a cohort study. Lancet. 369, 37-42.

Wilcken, B., Hammond, J., Silink, M., 1994. Morbidity and mortality in medium chain acyl coenzyme A dehydrogenase deficiency. Arch. Dis. Child. 70, 410-412.

Figure Legends

Figure 1. Effect of octanoic acid (OA) on oxygen consumption in non-ADP-stimulated (state 4) *(A)*, ADP-stimulated (state 3) *(B)*, respiratory control ratio (RCR) *(C)* and on ADP/O ratio *(D)* in mitochondria supported by glutamate/malate or succinate. After addition of mitochondria (0.5 mg . mL^{-1}), different concentrations of OA (0.5-3.0 mM) were added to the incubation medium. Values are means ± standard deviation for four independent experiments and states 3 and 4 of mitochondrial respiration are expressed as nmol O_2. min^{-1}. mg of $protein^{-1}$. **P*<0.05 ***P*<0.01, ****P*<0.001 compared to controls (Duncan multiple range test).

Figure 2. Effect of decanoic acid (DA) on oxygen consumption in non-ADP-stimulated (state 4) *(A)*, ADP-stimulated (state 3) *(B)*, respiratory control ratio (RCR) *(C)* and on ADP/O ratio *(D)* in mitochondria supported by glutamate/malate or succinate. After addition of mitochondria (0.5 mg . mL^{-1}), different concentrations of DA (0.5-3.0 mM) were added to the incubation medium. Values are means ± standard deviation for four independent experiments and states 3 and 4 of mitochondrial respiration are expressed as nmol O_2. min^{-1}. mg of $protein^{-1}$. **P*<0.05 ***P*<0.01, ****P*<0.001 compared to controls (Duncan multiple range test).

Figure 3. Effect of octanoic (OA) and decanoic (DA) acids on mitochondrial membrane potential using glutamate/malate *(A)* and *(C)* or succinate *(B)* and *(D)* as substrates. After addition of mitochondria (0.5 mg . mL^{-1}), OA and DA were added at 0.5-3.0 mM

concentrations. CCCP (1 μM) was added at the end of the measurements. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU).

Figure 4. Effect of octanoic (OA) and decanoic (DA) acids on oxygen consumption in uncoupled mitochondria (state U) using glutamate/malate as substrates. After addition of mitochondria (0.5 mg . mL^{-1}), 3 mM OA or DA were added to the incubation medium in the presence of 1 μM CCCP. Values are means ± standard deviation for four independent experiments and expressed as nmol O_2. min^{-1}. mg of $protein^{-1}$. $^{**}P<0.01$, $^{***}P<0.001$ compared to controls (Duncan multiple range test).

Figure 5. Effect of 3 mM octanoic (OA) and decanoic (DA) acids on the activities of the mitochondrial respiratory chain complexes I-III *(A)*, II-III *(B)* and IV *(C)*. Values are means ± standard deviation for four independent experiments and expressed as nmol . min^{-1}. mg of $protein^{-1}$. $^{**}P<0.01$, $^{***}P<0.001$ compared to controls (Duncan multiple range test).

Figure 6. Effect of atractyloside (ATC) on octanoic (OA) and decanoic (DA) acids-elicited stimulation of oxygen consumption in glutamate/malate-supported mitochondria respiring in state 4. After addition of mitochondria (0.5 mg . mL^{-1}), 3 mM OA or DA were supplemented to the incubation medium in the presence or in the absence of 1 μM ATC. Values are means ± standard deviation for four independent experiments and state 4 of mitochondrial respiration is expressed as nmol O_2. min^{-1}. mg of $protein^{-1}$. $^{**}P<0.01$, $^{***}P<0.001$ compared to controls (Duncan multiple range test).

Figure 7. Effect of octanoic (OA) *(A)* and decanoic (DA) *(B)* acids on mitochondrial NAD(P)H content using glutamate/malate as substrates. After addition of mitochondria (0.5 mg . mL^{-1}), OA or DA were given in 3.0 mM concentration. Rotenone (4 μM) was added at the end of the measurements. Data were expressed as nmol NAD(P)H . mg of $protein^{-1}$. Traces are representative of four independent experiments.

Figure 8. Effect of octanoic (OA) *(A)* and decanoic (DA) *(B)* acids on mitochondrial swelling. After addition of mitochondria (0.5 mg . mL^{-1}), OA or DA were given in 3.0 mM concentration. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU).

Figure 1.

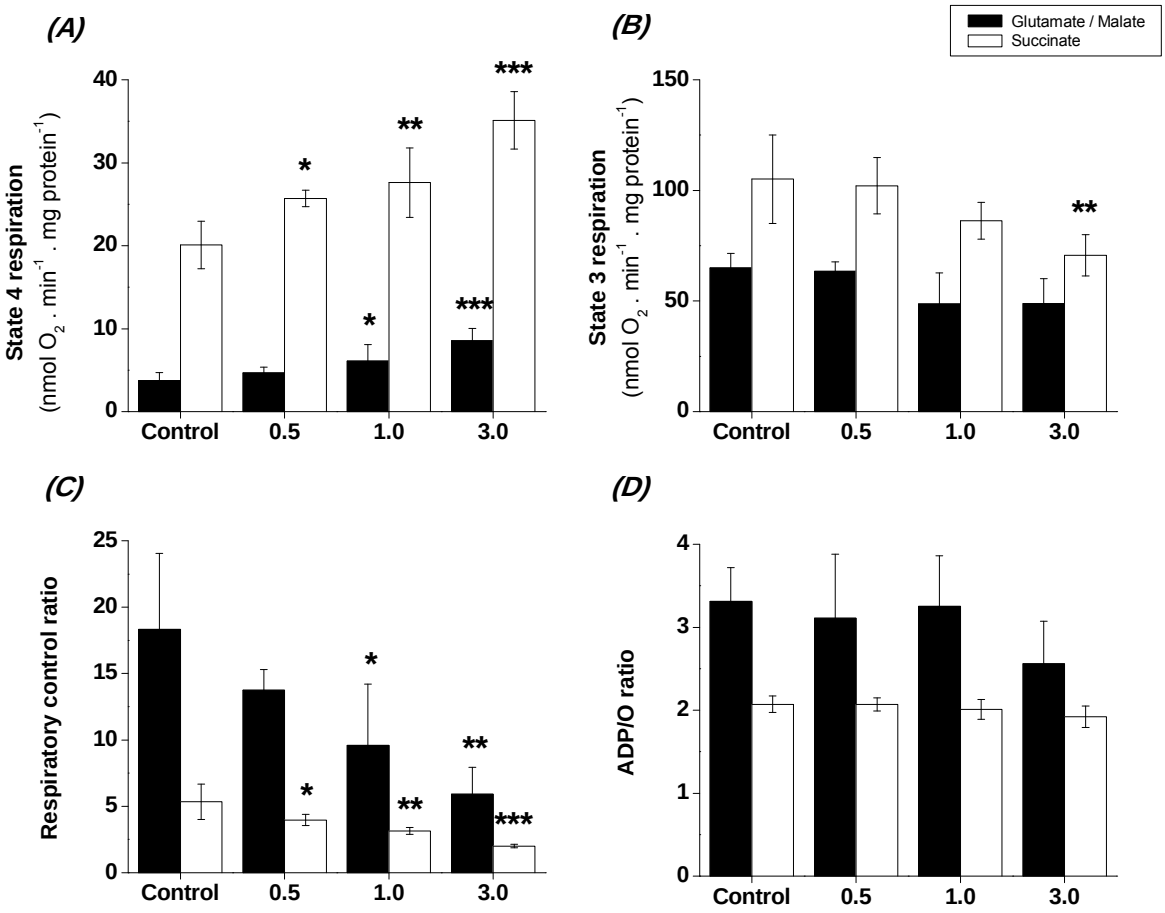

(A)
State 4 respiration
(nmol O2 . min-1 . mg protein-1)
40
30
20
10
0
Control
0.5
1.0
3.0
*
**
***
(B)
Glutamate / Malate
Succinate
State 3 respiration
(nmol O2 . min-1 . mg protein-1)
150
100
50
0
**
(C)
Respiratory control ratio
25
20
15
10
5
0
(D)
ADP/O ratio
4
3
2
1
0

Figure 2.

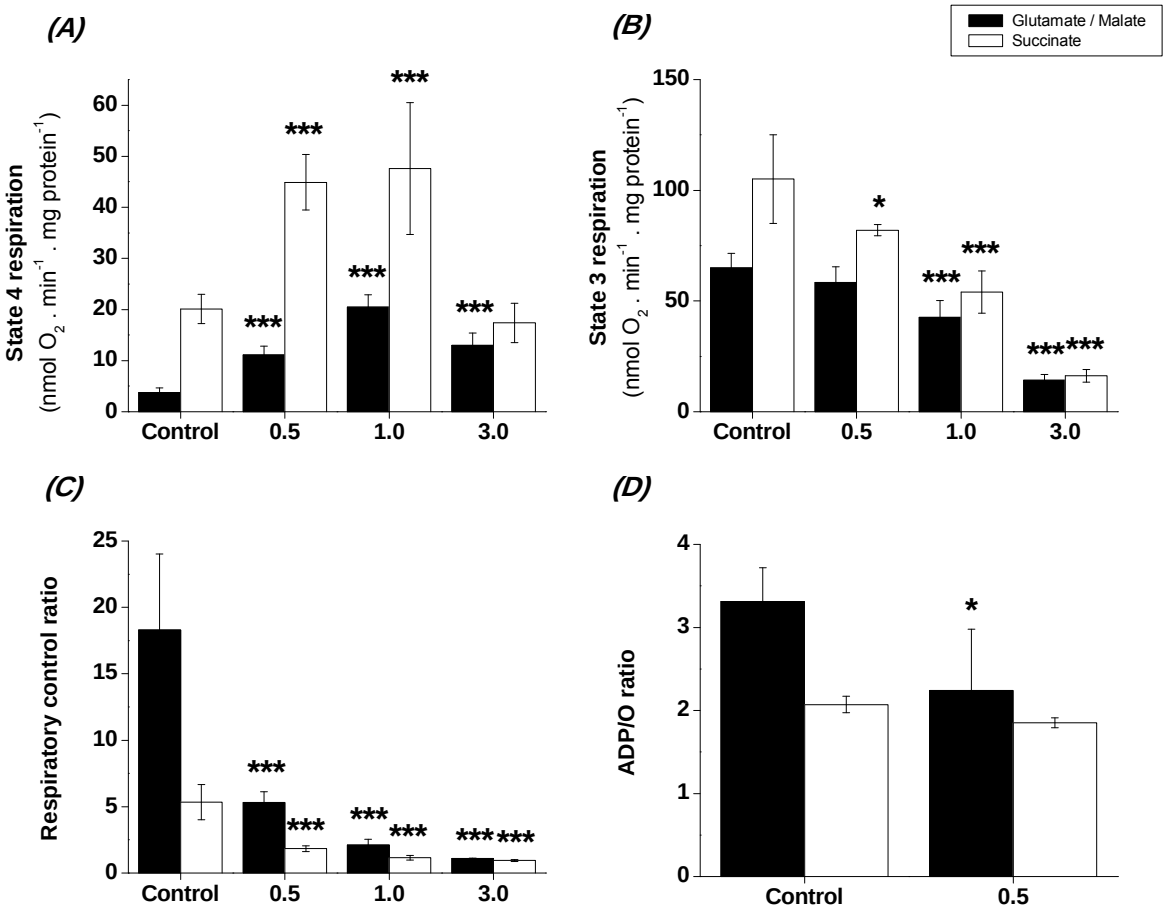

(A)
State 4 respiration
(nmol O_2 . min^{-1} . mg $protein^{-1}$)
Control
0.5
1.0
3.0
(B)
Glutamate / Malate
Succinate
State 3 respiration
(nmol O_2 . min^{-1} . mg $protein^{-1}$)
(C)
Respiratory control ratio
(D)
ADP/O ratio

Figure 3.

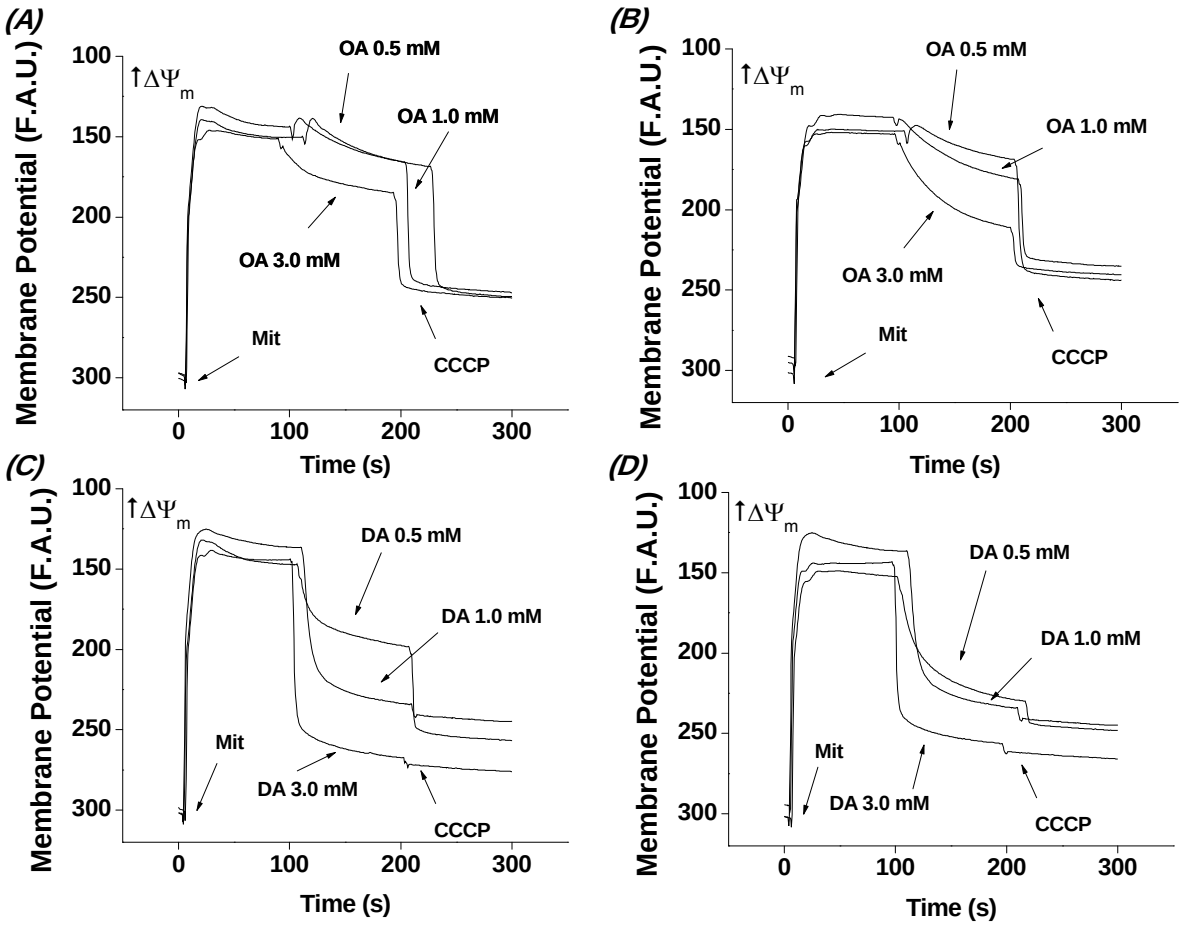

(A)
(B)
(C)
(D)
Membrane Potential (F.A.U.)
Time (s)
↑ΔΨm
OA 0.5 mM
OA 1.0 mM
OA 3.0 mM
DA 0.5 mM
DA 1.0 mM
DA 3.0 mM
Mit
CCCP
100
150
200
250
300
0
100
200
300

Figure 4.

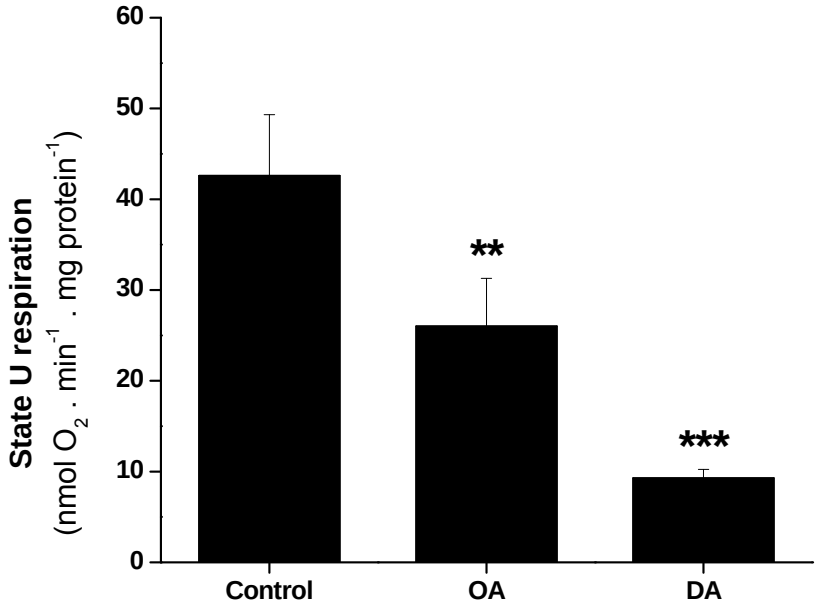

State U respiration
(nmol O_2 . min^{-1} . mg $protein^{-1}$)
60
50
40
30
20
10
0
**
***
Control
OA
DA

Figure 5.

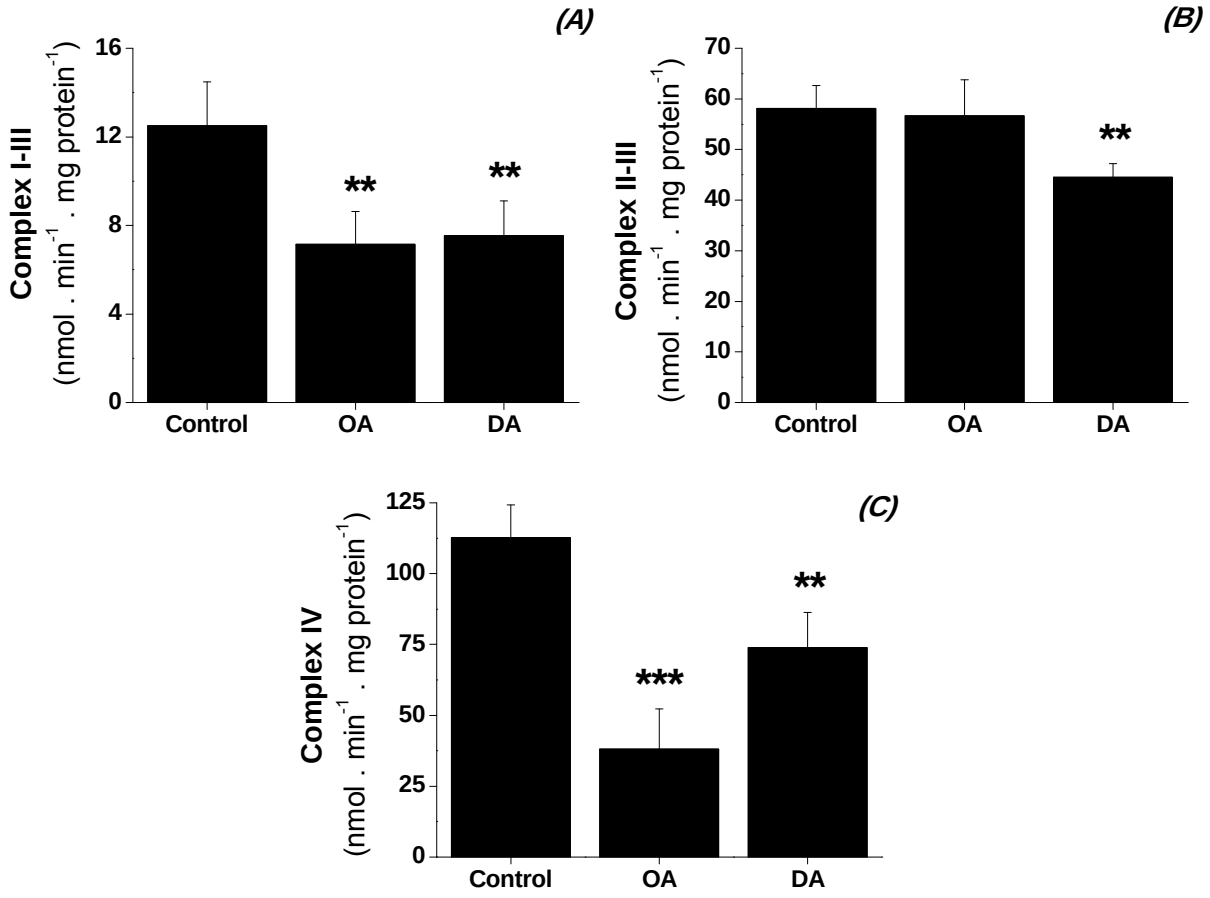

(A)
Complex I-III
(nmol . min-1 . mg protein-1)
16
12
8
4
0
**
**
Control
OA
DA
(B)
Complex II-III
(nmol . min-1 . mg protein-1)
70
60
50
40
30
20
10
0
**
Control
OA
DA
(C)
Complex IV
(nmol . min-1 . mg protein-1)
125
100
75
50
25
0
***
**
Control
OA
DA

Figure 6.

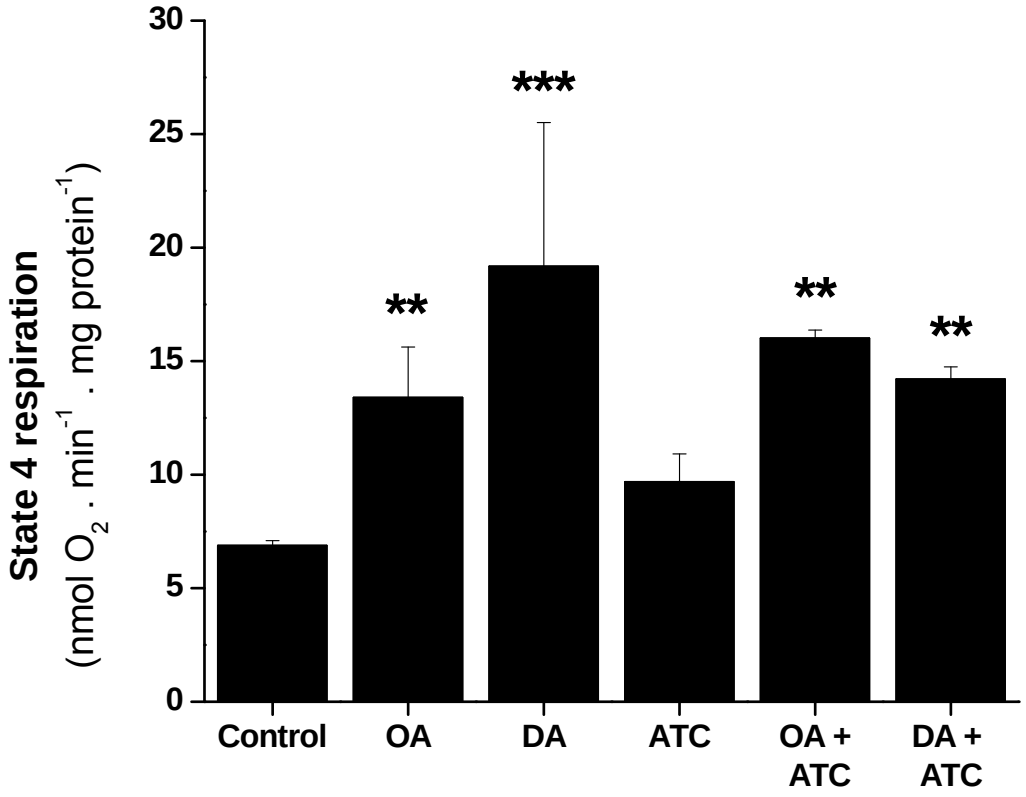

State 4 respiration
(nmol O_2 . min^{-1} . mg $protein^{-1}$)
30
25
20
15
10
5
0
***
**
**
**
Control
OA
DA
ATC
OA + ATC
DA + ATC

Figure 7.

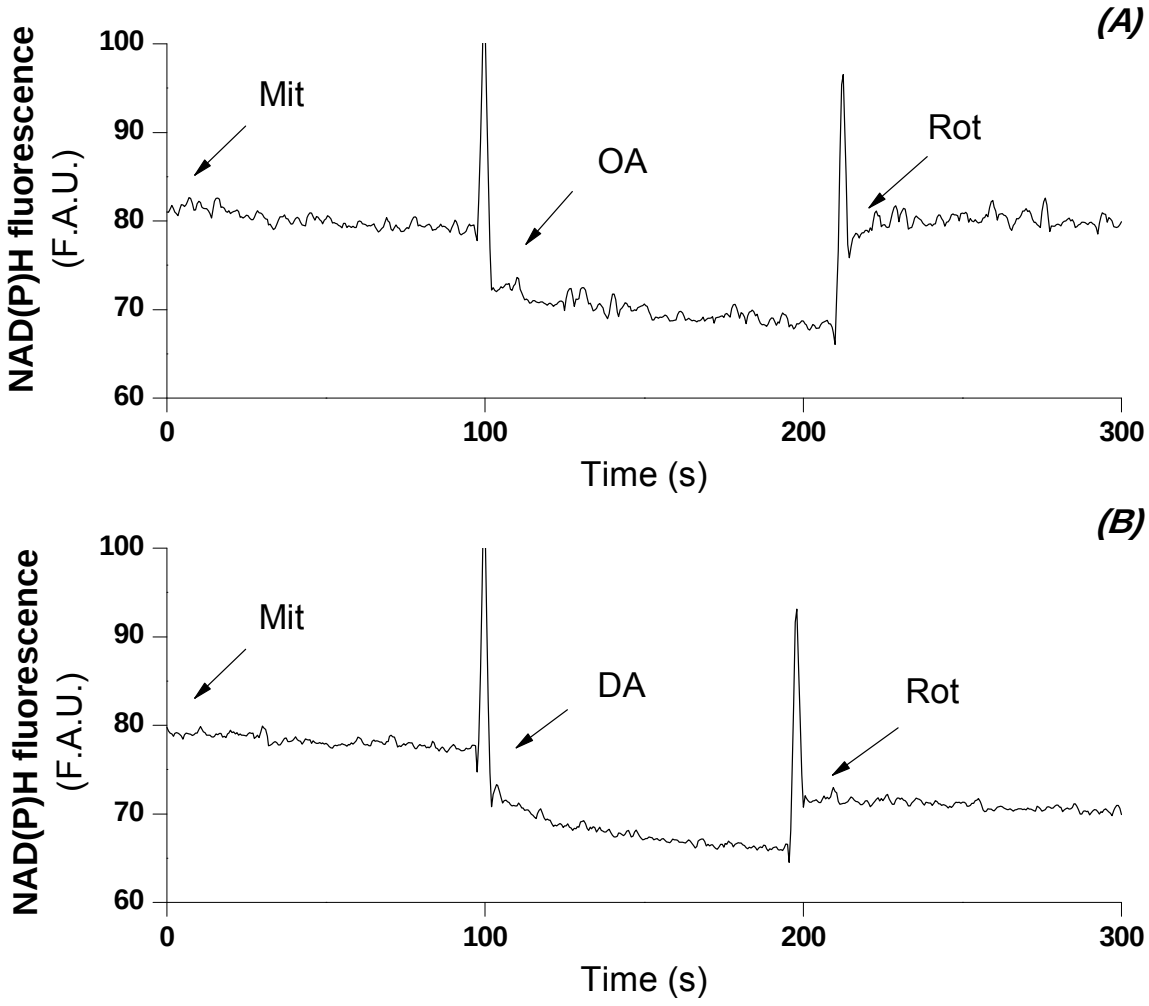
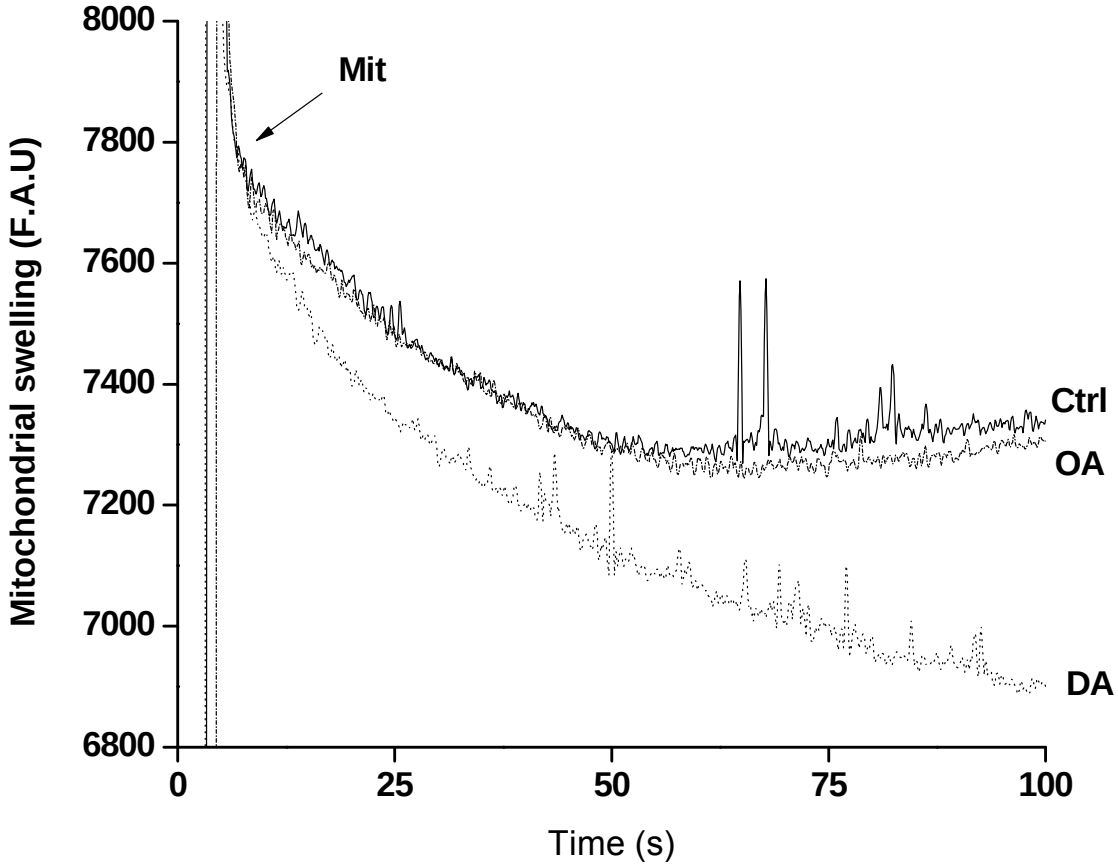

(A)
NAD(P)H fluorescence
(F.A.U.)
100
90
80
70
60
Mit
OA
Rot
0
100
200
300
Time (s)
(B)
NAD(P)H fluorescence
(F.A.U.)
100
90
80
70
60
Mit
DA
Rot
0
100
200
300
Time (s)

Figure 8.


8000
7800
7600
7400
7200
7000
6800
Mitochondrial swelling (F.A.U)
Mit
Ctrl
OA
DA
0
25
50
75
100
Time (s)

Capítulo III

Oxidative stress induction by cis-4-decenoic acid: Relevance for MCAD deficiency

Patrícia F. Schuck, Paula C. Ceolato, Gustavo C. Ferreira,

Anelise Tonin, Guilhian Leipnitz, Carlos S. Dutra-Filho,

Alexandra Latini, Moacir Wajner

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Oxidative stress induction by **cis**-4-decenoic acid: Relevance for MCAD deficiency

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Oxidative stress induction by *cis*-4-decenoic acid: Relevance for MCAD deficiency

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Abstract

Patients affected by medium-chain acyl-CoA dehydrogenase deficiency (MCADD) suffer from acute episodes of encephalopathy whose underlying mechanisms are poorly known. The present work investigated the *in vitro* effect of *cis*-4-decenoic acid (cDA), which accumulates in MCADD, on important parameters of oxidative stress in cerebral cortex of young rats. cDA markedly induced lipid peroxidation, as verified by the increased levels of spontaneous chemiluminescence and thiobarbituric acid-reactive substances. Furthermore, cDA significantly increased carbonyl formation and sulphydryl oxidation, which is indicative of protein oxidative damage, and promoted 2′,7′-dihydrodichlorofluorescein oxidation. It was also observed that the non-enzymatic tissue antioxidant defenses were decreased by cDA, whereas the antioxidant enzyme activities catalase, superoxide dismutase and glutathione peroxidase were not altered. Moreover, cDA-induced lipid peroxidation and GSH reduction was totally blocked by free radical scavengers, suggesting that reactive species were involved in these effects. The data indicate that oxidative stress is induced by cDA in rat brain *in vitro* and that oxidative damage might be involved in the pathophysiology of the encephalopathy in MCADD.

Keywords: *Reactive oxygen species, cis-4-decenoic acid, MCAD deficiency, lipid oxidation, protein oxidation, oxidative stress*

Introduction

Medium chain acyl-CoA dehydrogenase (MCAD; E.C. 1.3.99.3) deficiency (MCADD) is the most common inherited defect of fatty acid β-oxidation, with an approximate prevalence of 1:10 000 newborns [1,2]. The MCAD enzyme is responsible for the first step in mitochondrial β-oxidation of CoA esters of medium-chain fatty acids. A defect of this enzyme activity leads to predominant tissue accumulation of the medium-chain fatty acids octanoic (OA), decanoic (DA) and *cis*-4-decenoic (cDA), as well as their glycine and L-carnitine derivatives [3]. Clinical presentation of MCADD is related to fasting and other conditions with increased metabolic stress, that precipitate acute symptoms such as seizures, drowsiness or lethargy that may develop into coma or even sudden death [4]. Progressive encephalopathy with brain abnormalities is also found in this disorder [4–6]. Although most patients present during early infancy, some case reports have described neonatal [7–9] and adult presentations [10–12]. Treatment for ill patients consists of high amounts of glucose and L-carnitine administration during the acute episodes, as

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well as fat restriction, fasting avoidance and L-carnitine supplementation after recovery [2,13]. MCADD is often misdiagnosed as Reye syndrome because the neurological manifestations of these disorders are very similar [14]. Furthermore, OA accumulates in both MCADD and Reye syndrome, but the presence of cDA is pathognomonic of MCADD [15].

There are only few studies showing neurotoxic effects of OA, DA and cDA *in vitro* and *in vivo*. It was previously demonstrated that OA *in vivo* administration alter organic acid transport in rat choroid plexus, leading to impairment of the transchoroidal clearance of OA and similar compounds [16]. It was postulated that this effect may contribute to accumulation of medium-chain fatty acids (MCFA) in the brain and cerebral spinal fluid and possibly to the encephalopathy of patients affected by Reye syndrome. Other *in vitro* studies demonstrated that OA, DA and cDA were able to impair several parameters of energy metabolism in cerebral cortex of rats including the respiratory chain, mitochondrial creatine kinase and Na^+, K^+-ATPase activities [17–19], these effects being more pronounced with cDA. It was then presumed that this fatty acid may be the main neurotoxin in MCADD. Furthermore, since cDA was also able to elicit lipid peroxidation, it was suggested that reactive species generation could be responsible for the inhibitory effects of this fatty acid on the activities of these critical enzymes [19].

These observations led us to investigate in more details the *in vitro* effects of cDA on a wide spectrum of oxidative stress parameters, namely thiobarbituric acid-reactive substances (TBA-RS), spontaneous chemiluminescence, carbonyl content, sulphydryl oxidation, oxidation of mitochondrial membrane sulphydryl-bound protein groups (SBPG), 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation, total-radical trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), reduced glutathione (GSH) levels, as well as on the activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in homogenates from cerebral cortex of young rats in the hope to contribute to a better understanding of the mechanisms underlying the neurological dysfunction found in MCAD deficient patients. We also tested the role of antioxidants on some effects elicited by cDA.

Material and methods

Reagents

All chemicals were purchased from Sigma (St. Louis, MO), except for *cis*-4-decenoic acid (cDA) which was prepared by Dr Ernesto Brunet, Madrid, Spain, with 99% purity. cDA, decanoic acid (DA) and oleic acid (OLA) were dissolved on the day of the experiments in the incubation medium used for each technique with pH adjusted to 7.4. The final concentrations of the acids in the medium ranged from 0.1–1.0 mM.

Animals

A total of 92 30-day-old Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, were used. Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature ($22 \pm 1^\circ$C) colony room. The 'Principles of Laboratory Animal Care' (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

Tissue preparation and incubation

On the day of the experiments the animals were sacrificed by decapitation without anaesthesia and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum and striatum were discarded and the cerebral cortex was peeled away from the subcortical structures, weighed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at $750 \times g$ for 10 min at 4°C to discard nuclei and cell debris [20]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl at 37°C for 1 h with cDA at concentrations of 0.1, 0.5 or 1 mM, 1.0 mM DA or 1 mM OLA. Controls did not contain this metabolite in the incubation medium. Immediately after incubation, aliquots were taken to measure the values of TRAP, TAR, GSH, TBA-RS, chemiluminescence, carbonyl and sulphydryl content, DCFH oxidation, as well as the activities of CAT, SOD and GPx.

Oxidation of protein-bound sulphydryl (thiol) groups (PBSG) was studied in purified mitochondrial membrane preparations. The dissected cerebral cortex was homogenized (1:5 w/v) in 10 mM Tris, pH 7.4 containing 0.25 m sucrose. The homogenates were centrifuged at $650 \times g$ for 10 min to discard nuclei and cell debris. After a new centrifugation at $25\,000 \times g$ for 15 min, the resulting pellet containing the mitochondria was homogenized in 0.1 m Tris, pH 8.0 and the aliquots were stored at -70°C. On the day of the experiment, the aliquots were frozen/thawed three times and centrifuged at $15\,000 \times g$

for 2 min to collect the mitochondrial membranes. The resulting pellet was washed three times with 6.5% TCA with centrifugation at 15 000 × *g* for 2 min. The final pellet was resuspended in 0.5 m Tris buffer, pH 8.3, containing 0.5 mM EDTA and used to measure PBSG content.

Preparation of mitochondrial fractions

Mitochondrial fractions were prepared from cerebrum (total brain excluding cerebellum, olfactory bulbs, pons and medulla) of 30-day-old rats. The cerebrum was homogenized in 10 volumes of 5 mM potassium phosphate buffer, pH 7.4, containing 0.3 m sucrose, 5 mM MOPS, 1 mM EGTA and 0.1% bovine serum albumin. The homogenate was centrifuged at 1 500 × *g* for 10 min at 4°C and the pellet was discarded. The supernatant was then centrifuged for a further 10 min at 4°C at 15 000 × *g* in order to isolate the mitochondria present in the pellet, which was finally suspended in the same buffer. Disrupted mitochondrial fractions obtained by freezing/thawing three times were incubated at 37°C for 1 h with cDA at concentrations of 1.0 mM. Immediately after incubation, aliquots were used to measure TBA-RS.

Isolation of sub-mitochondrial particles

Sub-mitochondrial particles were prepared at 4°C from frozen and thawed mitochondria (20 mg protein/ml) according to Poderoso et al. [21]. The obtained sub-mitochondrial particles were washed twice with 140 mM KCl, 20 mM Tris-HCl, pH 7.4, and suspended in the same medium. The suspended particles were then incubated at 37°C for 1 h with 1 mM cDA. Immediately after incubation, aliquots from this preparation were used to measure superoxide formation.

Thiobarbituric acid-reactive substances (TBA-RS) levels

TBA-RS was determined according to the method of Esterbauer and Cheeseman [22]. Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of cDA pre-treated cerebral cortex supernatants and centrifuged at 300 × *g* for 10 min. Three hundred microlitres of the supernatant were transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulphate in a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool in running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as supernatants. Some experiments were performed in the presence or absence of reduced glutathione (GSH; 100 μm or 1 mM), melatonin (MEL; 200 μm or 1 mM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 500 μm or 1 mM), trolox (TRO; 1.5 μM or 10 μM), desferoxamine (DFO; 250 nM), L-carnitine (CAR; 1 mM), catalase (CAT; 2.5 mU/mL[1] or 10 mU/mL) plus superoxide dismutase (SOD; 2.5 mU/mL or 10 mU/mL), GM1 (10 μM), taurine (TAU; 1 mM) or creatine (Cr; 3 mM). Doses of these antioxidants were chosen according to previous works [22–24]. TBA-RS values were calculated as nmol of TBA-RS/mg protein and expressed as percentage of control.

Chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room by the method of Gonzalez et al. [23]. Incubation flasks contained 3.5 mL of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl were counted for background chemiluminescence during 5 min. An aliquot of 500 μL of cortical supernatant was immediately added and chemiluminescence was measured for 30 min at room temperature. The background chemiluminescence was subtracted from the total value and the results were calculated as cpm/mg protein and expressed as percentage of control.

Determination of protein carbonyl formation content

PCF (protein carbonyl content formation), a marker of oxidized proteins, was measured spectrophotometrically according to Reznick and Packer [24]. One hundred microlitres of the aliquots from the incubation were treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank control) and left in the dark for 1 h. Samples were then precipitated with 500 μL 20% TCA and centrifuged for 5 min at 10 000 × *g*. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and re-dissolved in 550 μL 6 m guanidine prepared in 2.5 N HCl Then, the tubes were incubated at 37°C for 5 min to assure the complete dissolution of the pellet and the resulting sample was determined at 365 nm. The difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl content. The results were calculated as nmol of carbonyls groups/mg of protein, using the extinction coefficient of 22 000 × 106 nmol/mL for aliphatic hydrazones.

2′,7′-dihydrodichlorofluorescein (DCFH) oxidation

Reactive species production was assessed according to LeBel et al. [25] by using 2′,7′-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl was incubated with the pre-treated cerebral cortex supernatants during 30 min at 37°C.

DCF-DA is enzymatically hydrolysed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of reactive species (RS). The DCF fluorescence intensity parallels to the amount of RS formed. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (0.25–10 μM) and the levels of RS were calculated as pmol DCF formed/mg protein.

Superoxide content

Superoxide production was determined spectrophotometrically according to Poderoso et al. [21] after exposition of sub-mitochondrial particles to 1 mM cDA. The assay is based on superoxide-dependent oxidation of epinephrine to adenochrome at 37°C ($E_{480nm} = 4.0$ mM/cm). The reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, pH 7.4, 0.1 mM catalase, 1 mM epinephrine and 7 mM succinate. Superoxide dismutase was used at 0.1–0.3 mM final concentrations as a negative control to confirm assay specificity.

Total radical-trapping antioxidant potential (TRAP)

TRAP, representing the total non-enzymatic antioxidant capacity of the tissue, was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. [26]. The reaction mixture containing 4 mL 10 mM ABAP dissolved in 0.1 m glycine buffer, pH 8.6 and 10 μL of luminol (4 mM) generates, at room temperature, an almost constant light intensity corresponding to free radical formation, which was measured in a Wallac 1409 liquid scintillation counter. This was considered to be the initial chemiluminescence values. Then, 30 μL of 300 μM trolox (soluble α-tocopherol analogue) or 30 μL of cortical supernatants were added to the reaction medium. The addition of trolox or supernatants provokes a marked reduction of the light intensity, which is maintained for a certain period after which light intensity rapidly increase. This period corresponds to induction time (IT) and represents TRAP measurement. IT is directly proportional to the antioxidant capacity of the tissue and the IT of each sample was compared with the IT of trolox. TRAP values were expressed as nmol trolox/mg of protein.

Total antioxidant reactivity (TAR)

TAR, which represents the quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by ABAP according to the method of Lissi et al. [27]. The chemiluminescence value was measured after 1 min after adding 4 mL 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) and 10 μL of luminol into a glass scintillation vial (initial chemiluminescence). Ten microlitres of 10–100 μM trolox (calibration curve) or brain supernatants, which decrease light intensity, were then added and chemiluminescence was measured after 60 s (final chemiluminescence). The ratio between the initial and the final chemiluminescence values is used to calculate TAR measurement. TAR values were expressed as nmol trolox/mg of protein.

Reduced glutathione (GSH) content

Reduced glutathione (GSH) concentrations were measured according to Browne and Armstrong [28]. cDA pre-treated cerebral cortex supernatants were diluted in 20 volumes of (1:20, v/v) 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microlitres of this preparation were incubated with an equal volume of *o*-phthaldialdehyde (1 mg/mL methanol) at room temperature during 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Some experiments were performed in the presence or absence of melatonin (MEL; 1 mM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 1 mM), trolox (TRO; 10 μM) or catalase (CAT; 2.5 mU/mL or 10 mU/mL) plus superoxide dismutase (SOD; 2.5 mU/mL or 10 mU/mL). Calibration curve was prepared with standard GSH (0.01–1 mM) and the concentrations were calculated as nmol/mg protein.

The oxidation of a commercial solution of GSH (1 mM) was also tested by exposing this solution to 1.0 mM cDA for 60 min in a medium devoid of brain supernatants. After cDA exposition, 7.4 mM *o*-phthaldialdehyde was added to the vials and the mixture was incubated at room temperature during 15 min.

Sulphydryl (thiol) group oxidation

This assay is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm [29]. Briefly, 40 μL of mitochondrial membrane suspension or 120 μL of cortical supernatant were incubated at 37°C for 1 h with cDA at concentrations of 0.1, 0.5 and 1 mM. Then, 1 mL of 0.1 mM DTNB was added. This was followed by 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Some experiments were carried out in the presence of 1 mM decanoic (DA) or 1 mM oleic (OLA) acids. The protein-bound sulphydryl content is inversely correlated to oxidative damage to

proteins. Results were reported as nmol TNB/mg protein.

Catalase (CAT) activity

CAT activity was assayed according to Aebi [30] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing 0.1–0.3 mg protein/mL. One unit (U) of the enzyme is defined as 1 mol of H_2O_2 consumed per minute. The specific activity was expressed as U/mg protein.

Superoxide dismutase (SOD) activity

SOD activity was determined according to Bannister and Calabrese [31] using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 32°C. Absorption was measured at 480 nm (4.0/mMcm). The reaction medium consisted of 50 mM glycine buffer pH 10.2, 0.1 mM catalase and 1 mM epinephrine. SOD specific activity is represented as U/mg protein.

Glutathione peroxidase (GPx) activity

GPx activity was measured according to Wendel [32] using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/ethylenediaminetetraacetic acid 1 mM, pH 7.7, 2 mM, glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH and the supernatant containing 0.2–0.3 mg protein/mL. One GPx unit (U) is defined as 1 mol of NADPH consumed per minute. The specific activity was calculated as mU/mg protein.

Protein determination

Protein was measured by the method of Lowry et al. [33] using bovine serum albumin as standard.

Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analysed 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 effects and correlations tests, linear regression was used. Only significant F-values are shown in the text. Differences between groups were rated significant at $p < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Results

cDA increased lipid peroxidation in rat cerebral cortex

We initially tested the influence of cDA on the lipid peroxidation parameters TBA-RS levels and chemiluminescence. We used a total of 12 animals for these experiments. Figure 1 shows that when cortical homogenates were exposed for 60 min to cDA, at 0.5 mM and 1 mM concentrations, TBA-RS levels significantly increased up to 58% ($F_{(3,20)} = 32.8$; $p < 0.001$) in a dose-dependent manner ($\beta = 0.87$; $p < 0.001$). Similarly, we found that cDA significantly increased the chemiluminescence values up to 39% ($F_{(3,20)} = 5.26$; $p < 0.01$). These results indicate that *in vitro* lipid peroxidation was increased in cerebral cortex exposed to cDA.

Next, we incubated cerebral cortex homogenates from four rats during 15, 30, 45 or 60 min in the presence or absence of cDA (0.1–1.0 mM) and observed a significant stimulatory effect of 1.0 mM cDA on lipid peroxidation (TBA-RS) at 30, 45 and 60 min incubation and at 60 min incubation with 0.5 mM cDA (Figure 2).

We also pre-incubated brain cortical homogenates for 15 min with reduced glutathione (GSH; 100 μM), melatonin (MEL; 200 μM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 500 μM), trolox (TRO; 1.5 μM), desferoxamine (DFO; 250 nM), L-carnitine (CAR; 1 mM), catalase (CAT; 2.5 mU/mL) plus superoxide dismutase (SOD; 2.5 mU/mL), GM1 (10 μM), taurine (TAU; 1 mM) or creatine (Cr; 3 mM). cDA (1.0 mM) was then added and incubation proceeded

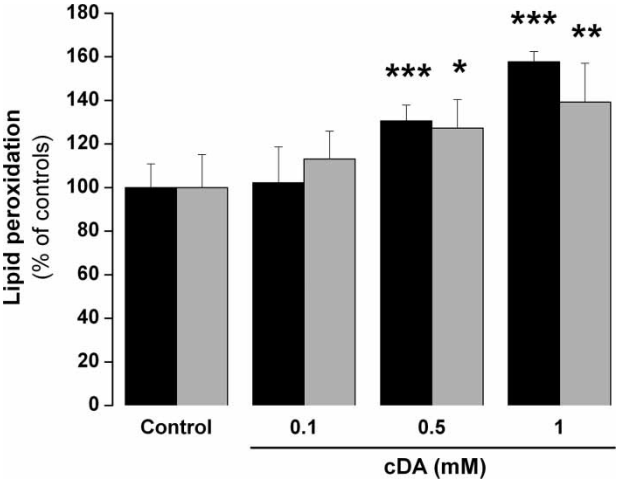

Figure 1. *In vitro* effect of *cis*-4-decenoic acid (cDA) on the lipid peroxidation parameters thiobarbituric acid-reactive substances (TBA-RS; black bars) and spontaneous chemiluminescence (gray bars) in rat cerebral cortex. Values are means ± standard deviation for six independent experiments performed in duplicate or triplicate and are expressed as % of controls (Controls: Chemiluminescence 1019 ± 154 cpm/mg protein; TBA-RS levels 3.42 ± 0.37 nmol/mg protein). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to controls (Duncan multiple range test).

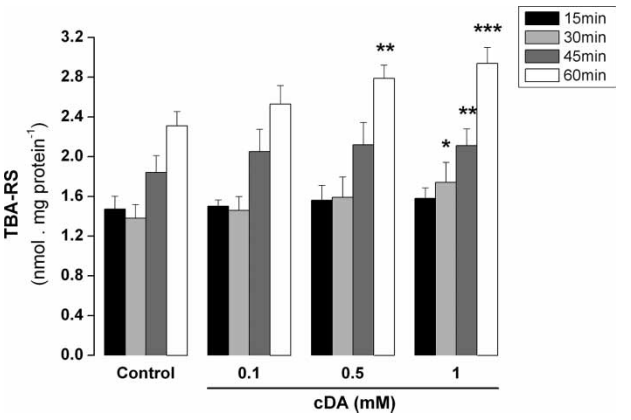

Figure 2. *In vitro* effect of exposition time on *cis*-4-decenoic acid (cDA) stimulatory effect on thiobarbituric-acid reactive substances (TBA-RS) in rat cerebral cortex. Cortical homogenates were incubated for 15, 30, 45 or 60 min with cDA (0.1–1.0 mM) and TBA-RS levels measured afterwards. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as nmol/mg protein. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to controls (Duncan multiple range test).

for 60 min, after which TBA-RS levels were measured. For these experiments a total of 15 rats were used. We can observe in Figure 3A that TAU, Cr and GM1, at the common doses used as antioxidants, prevented cDA-induced increased lipid peroxidation ($F_{(11,62)} = 3.35$; $p < 0.01$). In contrast, GSH, TRO, CAT plus SOD, MEL, L-NAME, CAR and DFO were not able to prevent cDA increase of TBA-RS values (results not shown). However, higher doses of GSH (1 mM), TRO (10 μM) and CAT plus SOD (10 mU/mL of each enzyme), but not MEL (1 mM) and L-NAME (1 mM), also prevented cDA-induced increase of TBA-RS ($F_{(11,55)} = 25.6$; $p < 0.001$) (Figure 3B).

We also verified that concentrations as high as 1.0 mM of cDA did not change TBA-RS levels in mitochondrial preparations, indicating that generation of oxidants by this organic acid causing lipid damage occurred via cytosolic rather than mitochondrial mechanisms (results not shown).

cDA induces protein oxidative damage in rat cerebral cortex

We also evaluated the effect of cDA on carbonyl formation and sulphydryl oxidation in cortical homogenates (protein oxidation). Brains from 11 animals were used in these experiments. We found that protein carbonyl groups were increased (up to 67%) in cortical homogenates in the presence of cDA ($F_{(3,16)} = 4.72$; $p < 0.05$) in a dose-dependent manner ($\beta = 0.68$; $p < 0.001$) (Figure 4A). cDA also induced significant sulphydryl oxidation (31%) at 1 mM ($F_{(3,18)} = 6.76$; $p < 0.01$) (Figure 4B), similarly to OLA which decreased the sulphydryl groups by ~54%. In contrast, DA did not alter this parameter.

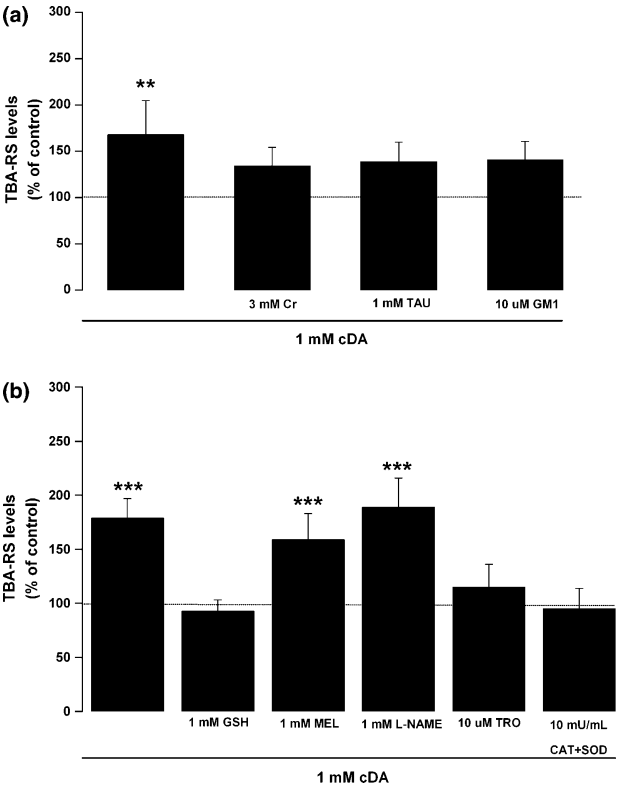

Figure 3. *In vitro* effect of creatine (Cr), taurine (TAU) and GM1 (A) and of the antioxidants GSH, MEL, L-NAME, TRO and CAT + SOD (B) on *cis*-4-decenoic acid (cDA)-induced increase of thiobarbituric-acid reactive substances (TBA-RS) in rat cerebral cortex. Cortical homogenates were pre-incubated for 15 min with the antioxidants before the addition of 1 mM cDA. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as % of control (Controls: 3.99 ± 0.67 nmol/mg protein). $**p < 0.01$; $***p < 0.001$ compared to controls (Duncan multiple range test).

cDA provokes oxidation of DCFH

Next, we assessed the influence of cDA on DCFH oxidation in cerebral cortex from six rats. We observed that cDA significantly increased (up to 28%) DCFH oxidation ($F_{(3,20)} = 7.86$; $p < 0.001$) in a dose-dependent manner ($\beta = 0.73$; $p < 0.001$) (Figure 5).

cDA does not affect superoxide generation

We also evaluated superoxide generation in submitochondrial particles from cerebral cortex supernatants exposed for 1 h to 1.0 mM cDA and observed that this fatty acid did not increase superoxide content (results not shown).

Reduction of brain non-enzymatic antioxidant defenses by cDA

We also investigated the *in vitro* effect of cDA on the non-enzymatic antioxidant defenses, by measuring TRAP, TAR and GSH levels. For these experiments a total of 22 rats were used. cDA, at concentrations of 0.5 mM and higher, significantly diminished TRAP,

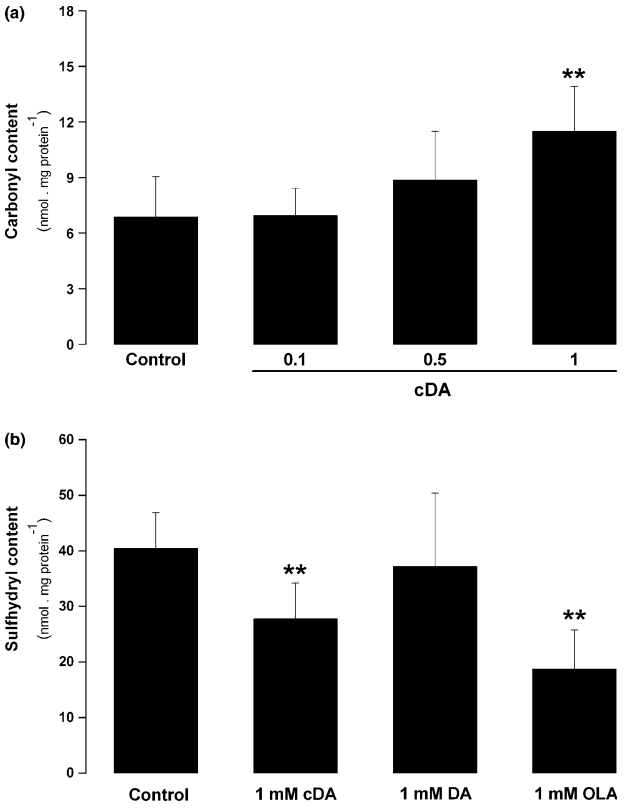

Figure 4. *In vitro* effect of *cis*-4-decenoic acid (cDA) on carbonyl (A) and sulphydryl content (B) in rat cerebral cortex. Values are means ± standard deviation for five independent experiments performed in triplicate and are expressed as nmol/mg protein. $^{**}p < 0.01$ compared to control (Duncan multiple range test).

TAR and GSH values (up to 35%) in rat cortical homogenates in a dose-dependent manner (TRAP: ($F_{(3,16)} = 8.87$; $p < 0.001$) ($\beta = 0.77$; $p < 0.001$); TAR: ($F_{(3,28)} = 10.7$; $p < 0.001$) ($\beta = 0.71$; $p < 0.001$); GSH: ($F_{(3,32)} = 14.2$; $p < 0.001$) ($\beta = 0.75$; $p < 0.001$)) (Figure 6). It was also observed that cDA-induced GSH reduction was totally prevented by the scavengers MEL and TRO, but not by CAT plus SOD ($F_{(4,29)} = 13.8$; $p < 0.001$). Taken together, the data indicate a marked reduction of the brain non-enzymatic antioxidant defenses by cDA. Furthermore, cDA-induced reduction of TRAP (total tissue non-enzymatic antioxidant defenses) was inversely correlated with TBA-RS values (a lipid peroxidation parameter) ($\beta = -0.69$; $p < 0.001$), suggesting that free radicals inducing lipid peroxidation were probably responsible for the decrease of the non-enzymatic antioxidant defenses (Figure 7).

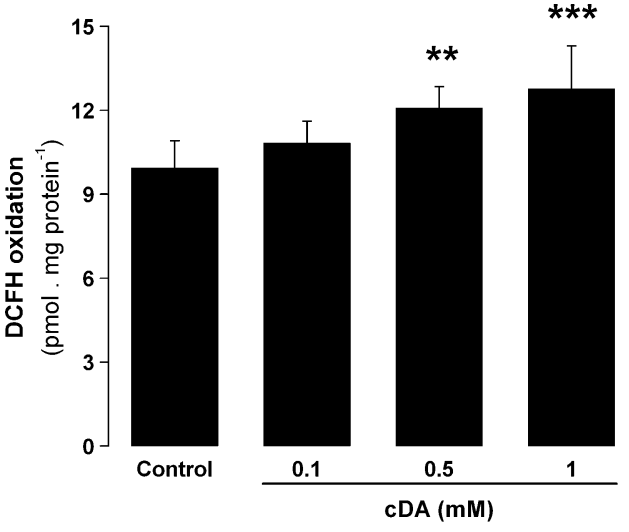

Figure 5. *In vitro* effect of *cis*-4-decenoic acid (cDA) on DCFH oxidation in rat cerebral cortex. Values are means ± standard deviation for six independent experiments performed in triplicate and are expressed as pmol/mg protein. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control (Duncan multiple range test).

The next set of experiments was designed to investigate whether the cDA-induced decrease of brain GSH levels was due to a direct oxidative attack, rather than due to promotion of free radicals. We therefore exposed commercial GSH, as well as membrane protein-bound sulphydryl groups (PBSG) purified from mitochondrial fractions to 0.1–1.0 mM cDA in the absence of tissue supernatants. It was observed that cDA by itself did not oxidize free GSH or PBSG, suggesting that cDA *per se* is not a direct oxidant agent, but elicits oxidative damage probably via free radical generation (Table I).

Antioxidant enzyme activities were not affected by cDA

Finally, the *in vitro* effect of cDA on catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were examined by pre-incubating cortical homogenates for 60 min with cDA (0.1, 0.5 and 1.0 mM cDA). For these experiments we used 12 animals. We did not verify any alteration on CAT, SOD and GPx activities, suggesting that cDA did not affect these brain antioxidant enzymes (Table II).

Discussion

Patients with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (MCADD) present acute episodes of decompensation characterized by severe hypoketotic hypoglycaemia, acidosis, hyperammonaemia, vomiting, hypotonia, coma, seizures or even death, which occurs in about 20–25% of the affected individuals during the first episode [4,34–37]. Furthermore, a considerable number of those who survive present a variable degree of neurological dysfunction with cerebral abnormalities [5,6,10,38–40], whose pathophysiology is not yet defined. In this context, although high ammonia levels and hypoglycaemia could be tentatively related to the neurological dysfunction in these patients, the moderate degree of these laboratorial alterations usually do not lead to disturbances of neural function. Therefore, it could be presumed that the increased tissue concentrations of the medium-chain fatty acid octanoate, decanoate

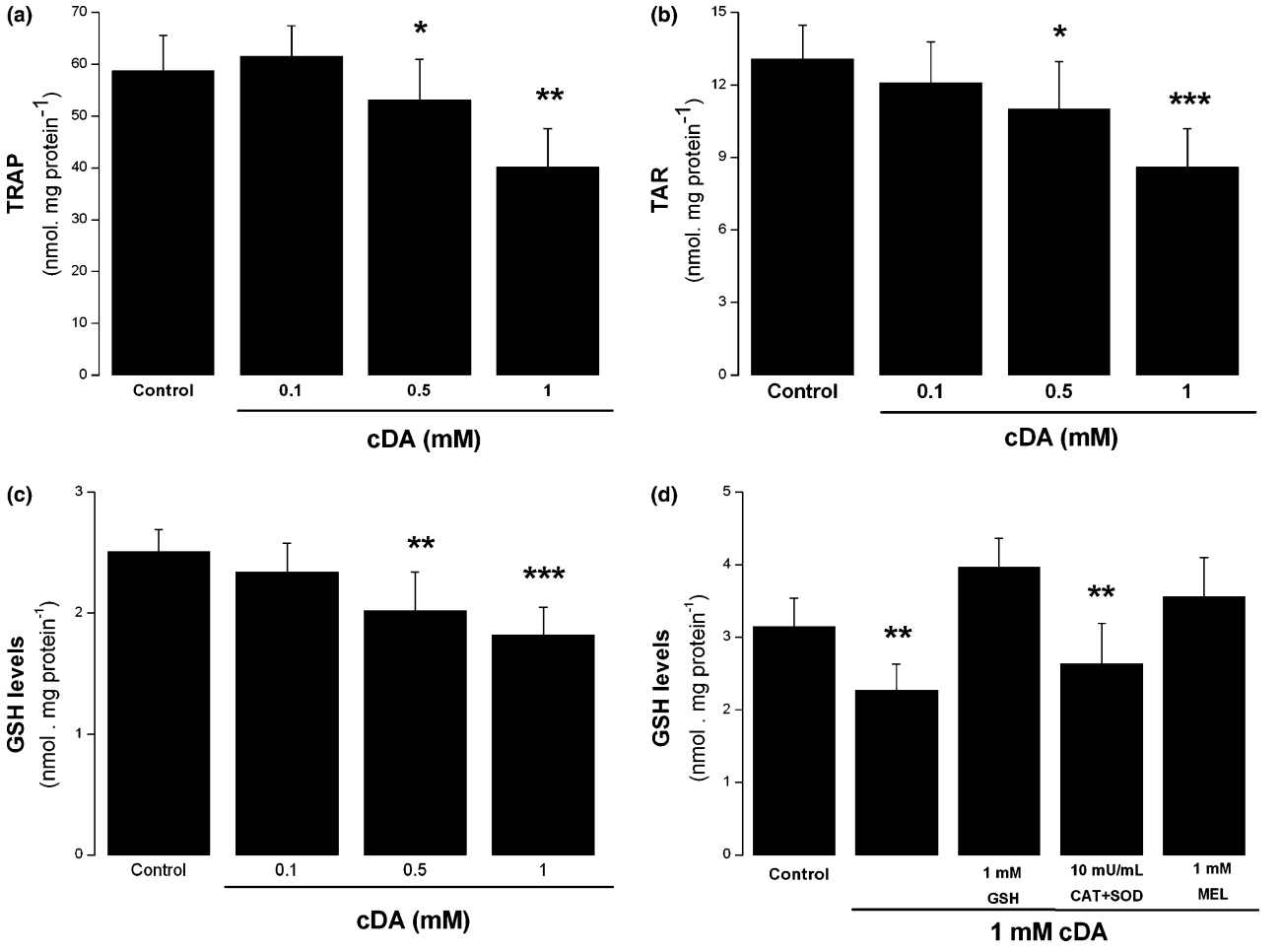

Figure 6. *In vitro* effect of *cis*-4-decenoic acid (cDA) on the non-enzymatic antioxidant parameters total-radical trapping antioxidant potential (TRAP) (A), total antioxidant reactivity (TAR) (B) and reduced glutathione (GSH) levels (C) in rat cerebral cortex. The effects of cDA on GSH levels in the presence of the antioxidants MEL, TRO and CAT plus SOD were also tested (D). Values are means ± standard deviation for five-to-nine independent experiments performed in duplicate or triplicate and are expressed as nmol/mg protein. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to controls (Duncan multiple range test).

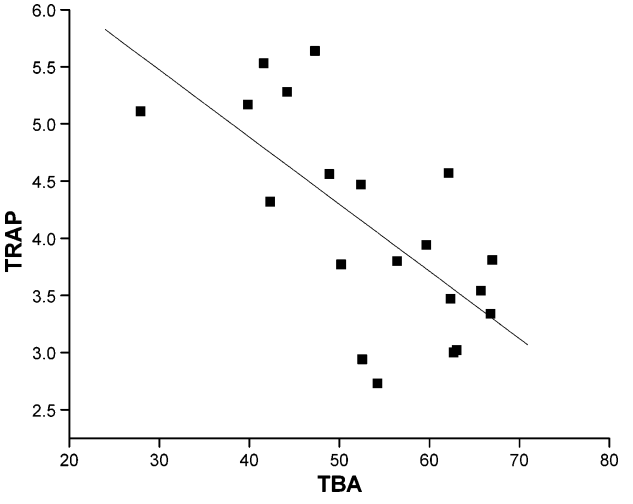

Figure 7. Correlation between thiobarbituric acid-reactive substances (TBA-RS) and total-radical trapping antioxidant potential (TRAP) in rat cerebral cortex exposed to 0.1–1.0 mM *cis*-4-decenoic acid (cDA). Values are means ± standard deviation for five-to-six independent experiments performed in triplicate and are expressed as nmol/mg protein for TBA-RS and TRAP.

Table I. Effect of *cis*-4decenoic acid (cDA) on membrane protein-bound sulphydryl groups (PBSG) purified from mitochondrial fractions of rat cerebral cortex and on commercial reduced glutathione (GSH).

Mitochondrial PBSG oxidation	
Control	63.0±8.72
0.1 mM cDA	63.9±6.16
0.5 mM cDA	59.8±8.33
1.0 mM cDA	54.9±5.76
GSH oxidation	
Control	100±4.04
1.0 mM cDA	116±7.93

Values are means±standard deviation for three-to-six independent experiments performed in triplicate and are expressed as nmol TNB/mg protein (mitochondrial PBSG oxidation) and percentage of control (GSH oxidation; controls: 1125±47.4 unidades de fluorescência). No significant differences were detected (One-way ANOVA).

(DA) and *cis*-4-decenoate (cDA) that accumulate in MCADD may be responsible, at least in part, for the neurological symptoms in this disorder.

Considering that oxidative stress is involved in the pathophysiology of common neurodegenerative disorders [41–46] and of some inborn errors of metabolism [47–52], in the present study we evaluated the *in vitro* influence of cDA, the pathognomonic compound in MCADD, on several parameters of oxidative stress in cerebral cortex of young rats in order to evaluate whether this pathomechanism could be acting in this disorder. We first observed that cDA markedly increased TBA-RS (60%) and spontaneous chemiluminescence (40%). Furthermore, TBA-RS values gradually increased when cerebral cortex homogenates were exposed for increasing periods to cDA, suggesting that free radical synthesis was probably involved in this effect. Light emitted in the chemiluminescence assay usually arises from peroxidizing lipids due to an increase in reactive oxygen or nitrogen species production and TBA-RS reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation [53]. Therefore, our data strongly indicate that cDA induced lipid peroxidation in cerebral cortex *in vitro*.

We also observed that the strong *in vitro* lipid peroxidation caused by cDA was totally prevented by high doses of the free radical scavengers GSH, TRO and the combination of CAT plus SOD, but not by smaller doses of these antioxidants. Furthermore, Cr, TAU and GM1, but not DFO, MEL, L-NAME and CAR, also prevented cDA-induced increase of TBA-RS. These data suggest that cDA provoked a strong pro-oxidant effect on membrane lipids from cerebral cortex, indicating that the toxic effect exerted by cDA is mediated by reactive oxygen species generation. In contrast, cDA did not change TBA-RS levels measured in purified mitochondrial preparations (post-mitochondrial supernatants), suggesting that the generation of oxidants by this organic acid causing lipid oxidative damage occurred via cytosolic rather than mitochondrial mechanisms.

Table II. Effect of *cis*-4decenoic acid (cDA) on the antioxidant enzyme activities catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in rat cortical supernatants.

	CAT	SOD	GPx
Control	2.74±0.09	2.08±0.19	13.6±2.83
0.1 mM cDA	2.48±0.49	1.71±0.68	13.9±1.25
0.5 mM cDA	2.40±0.52	2.35±0.62	13.0±1.94
1.0 mM cDA	2.58±0.54	1.81±0.35	11.4±4.26

Values are means±standard deviation for four independent experiments performed in duplicate and are expressed as: CAT and SOD: U/mg protein and GPx: mU/mg protein. No significant differences were detected (One-way ANOVA).

Protein carbonyl formation and sulphydryl oxidation, useful markers for assessing oxidative protein damage, were also markedly increased by cDA, implying that this unsaturated carboxylic acid also provokes protein oxidation [24,55]. Similarly, the unsaturated oleic acid (OLA), but not decanoic acid (DA), a saturated fatty acid with the same carbon chain as cDA, significantly enhanced sulphydryl oxidation, suggesting that this effect may be related to the unsaturated bond. Previous findings showing that the polyunsaturated arachidonic acid induces reactive oxygen species reinforces this assumption [54]. On the other hand, the observations that cDA was not able to oxidize purified thiol groups from purified mitochondrial membranes as well as commercial reduced glutathione in the absence of brain supernatants in the incubation medium suggest that this fatty acid is not *per se* an oxidant agent and that it probably provoked lipid and protein oxidative damage via free radical induction.

With regard to the antioxidant defenses, cDA markedly reduced the total content of non-enzymatic antioxidants (TRAP values) and GSH, the main natural-occurring antioxidant, as well as the capacity to rapidly handle increased free radical generation (TAR measurement) in cerebral cortex. Since these parameters are used to evaluate the capacity of a tissue to prevent and respond to the damage associated to free radical processes, it can be concluded that the rat cortical antioxidant defenses were severely compromised by cDA [20,27,53]. We verified that MEL and TRO, but not SOD plus CAT, were able to prevent cDA-decrease of GSH levels in cortical supernatants, suggesting that the highly toxic hydroxyl radical, which is the principal species scavenged by MEL [56], and peroxyl radicals, which are scavenged by TRO, were probably involved in the reduction of GSH provoked by cDA.

We also observed that cerebral cortex homogenates exposed to cDA resulted in an enhanced DCFH oxidation, which is predominantly dependent on hydrogen peroxide [57]. These results allied to those showing that GSH, TRO and SOD plus CAT fully

prevented the stimulation of *in vitro* lipid peroxidation elicited by cDA suggest that the free radicals hydroxyl, peroxyl and hydrogen peroxide, which are scavenged by these antioxidants [58–61], are involved in the pro-oxidant effects of cDA. In contrast, we found that cDA did not affect superoxide generation in sub-mitochondrial particles obtained from cerebral cortex supernatants, ruling out an important involvement of this free radical in cDA pro-oxidant effects.

A significant inverse correlation between TBA-RS increase and TRAP decrease caused by cDA was also verified in the present study, reinforcing the view that the decrease of the brain antioxidant defenses and the induction of lipid peroxidation were mediated by increased reactive species generation. Therefore, it is feasible that the marked decline of the cerebral non-enzymatic antioxidant defenses (TRAP, GSH and TAR) probably reflects the rapid consume of highly reactive antioxidants due to the generation of free radicals elicited by cDA.

Although we cannot at the present establish the exact signal transduction cascades by which cDA induced lipid and protein oxidative damage in cerebral cortex, it may be presumed that free radicals elicited by this fatty acid could initiate the classical cascades leading to these pro-oxidant effects [62].

We also observed that the activities of the antioxidant enzymes CAT, GPx and SOD were not altered by exposing cortical supernatants for 60 min to cDA at concentrations as high as 1.0 mM. However, we cannot exclude the possibility that sustained high concentrations of cDA, as occurs in MCADD, could lead to up-regulation of these enzymes via gene transcription in order to counterbalance increased free radical generation, as occurs in other pathological conditions involving oxidative stress [63–65].

Since oxidative stress results from imbalance between the total antioxidant defense of the tissue and the reactive species generated, our present data strongly indicate that cDA provokes a significant *in vitro* stimulation of oxidative stress in cerebral cortex, a deleterious cell condition, which induces oxidation of lipids and proteins and reduces antioxidant defenses [53]. At this point, it should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues [66], a fact that makes this tissue more vulnerable to increased reactive species. In fact, oxidative stress has been implicated in the pathophysiology of common neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, as well as in epileptic seizures and demyelination [24,63,66,67].

We cannot establish whether our data have a pathophysiological significance, since brain concentrations of cDA in MCAD deficient patients are unknown. It should, however, be noted that the significant alterations of the oxidative stress parameters elicited by cDA occurred at high micromolar levels (0.50 mM and higher) and acute encephalopathy occurs in these patients particularly during metabolic crises, in which the concentrations of the accumulating metabolites dramatically increase. Furthermore, it has been proposed that the concentrations of organic acids accumulating in various organic acidemias are higher in the neural cells as compared to the serum or CSF [68].

In conclusion, the present data indicate that oxidative stress is induced by cDA in the brain of young rats. In case these present findings are confirmed *in vivo* in animal experiments and also in tissues of patients affected by MCADD, it is tempting to speculate that excessive reactive species generation might contribute, at least in part, to the neuropathology of this disorder. It seems therefore reasonable to propose that antioxidants may serve as an adjuvant therapy to specific diets or to other pharmacological agents used for these patients, especially during crises, to avoid oxidative damage to the brain.

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References

[1] Rinaldo P, Raymond K, Al-Odaib A, Bennett M. Clinical and biochemical features of fatty acid oxidation disorders. Curr Opin Pediatr 1998;10:615–621.

[2] Roe CR, Ding J. Mitochondrial fatty acid oxidation disorders. In: CR Scriver, AL Beaudet, WS Sly, D Valle, editors. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill; 2001. p 1909–1963.

[3] Martynez G, Jimenez-Sanchez G, Divry P, Vianey-Saban C, Riudor E, Rodes M, Briones P, Ribes A. Plasma free fatty acids in mitochondrial fatty acid oxidation defects. Clin Chim Acta 1997;267:143–154.

[4] Derks TG, Reijngoud DJ, Waterham HR, Gerver WJ, van den Berg MP, Sauer PJ, Smit GP. The natural history of medium-chain acyl CoA dehydrogenase deficiency in the Netherlands: clinical presentation and outcome. J Pediatr 2006;148:665–670.

[5] Wilson CJ, Champion MP, Collins JE, Clayton PT, Leonard JV. Outcome of medium chain acyl-CoA dehydrogenase deficiency after diagnosis. Arch Dis Child 1999;80:459–462.

[6] Mayell SJ, Edwards L, Reynolds FE, Chakrapani AB. Late presentation of medium-chain acyl-CoA dehydrogenase deficiency. J Inherit Metab Dis 2007;30:104.

[7] Leung KC, Hammond JW, Chabra S, Carpenter KH, Potter M, Wilcken B. A fatal neonatal case of medium-chain acyl-coenzyme A dehydrogenase deficiency with homozygous A- > G985 transition. J Pediatr 1992;121:965–968.

[8] Wilcken B, Carpenter KH, Hammond J. Neonatal symptoms in medium chain acyl coenzyme A dehydrogenase deficiency. Arch Dis Child 1993;69:292–294.

[9] Maclean K, Rasiah VS, Kirk EP, Carpenter K, Cooper S, Lui K, Oei J. Pulmonary haemorrhage and cardiac dysfunction in

a neonate with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. Acta Paediatr 2005;94:114–116.

[10] Ruitenbeek W, Poels PJ, Turnbull DM, Garavaglia B, Chalmers RA, Taylor RW, Gabreels FJ. Rhabdomyolysis and acute encephalopathy in late onset medium chain acyl-CoA dehydrogenase deficiency. J Neurol Neurosurg Psychiatry 1995;58:209–214.

[11] Raymond K, Bale AE, Barnes CA, Rinaldo P. Medium-chain acyl-CoA dehydrogenase deficiency: sudden and unexpected death of a 45 year old woman. Genet Med 1999;1:293–294.

[12] Feillet F, Steinmann G, Vianey-Saban C, de Chillou C, Sadoul N, Lefebvre E, Vidailhet M, Bollaert PE. Adult presentation of MCAD deficiency revealed by coma and severe arrythmias. Intensive Care Med 2003;29:1594–1597.

[13] Coates PM. New developments in the diagnosis and investigation of mitochondrial fatty acid oxidation disorders. Eur J Pediatr 1994;153:49–56.

[14] Santer R, Schmidt-Sommerfeld E, Leung YK, Fischer JE, Lebenthal E. Medium-chain acyl CoA dehydrogenase deficiency: electron microscopic differentiation from Reye syndrome. Eur J Pediatr 1990;150:111–114.

[15] Onkenhout W, Venizelos V, van der Poel PFH, Van der Heuvel MPM, Poorthuis BJHM. Identification and quantification of intermediates of unsaturated fatty acid metabolism in plasma of patients with fatty acid oxidation disorders. Clin Chem 1995;41:1467–1474.

[16] Kim CS, O'tuama LA, Mann JD, Roe CR. Effect of increasing carbon chain length on organic acid transport by the choroid plexus: a potential factor in Reye's syndrome. Brain Res 1983;259:340–343.

[17] de Assis DR, Ribeiro CA, Rosa RB, Schuck PF, Dalcin KB, Vargas CR, Wannmacher CM, Dutra-Filho CS, Wyse AT, Briones P, Wajner M. Evidence that antioxidants prevent the inhibition of Na+,K+-ATPase activity induced by octanoic acid in rat cerebral cortex *in vitro*. Neurochem Res 2003;28:1255–1263.

[18] Reis de Assis D, Maria RC, Borba Rosa R, Schuck PF, Ribeiro CA, da Costa Ferreira G, Dutra-Filho CS, Terezinha de Souza Wyse A, Duval Wannmacher CM, Santos Perry ML, Wajner M. Inhibition of energy metabolism in cerebral cortex of young rats by the medium-chain fatty acids accumulating in MCAD deficiency. Brain Res 2004;1030:141–151.

[19] de Assis DR, Maria RC, Ferreira GC, Schuck PF, Latini A, Dutra-Filho CS, Wannmacher CM, Wyse AT, Wajner M. Na+, K+ ATPase activity is markedly reduced by cis-4-decenoic acid in synaptic plasma membranes from cerebral cortex of rats. Exp Neurol 2006;197:143–149.

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

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

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

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

[24] Reznick AZ, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol 1994;233:357–363.

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

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

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

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

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

[30] Aebi H. Catalase *in vitro*. Methods Enzymol 1984;105:121–126.

[31] Bannister JV, Calabrese L. Assays for SOD. Methods Biochem Anal 1987;32:279–312.

[32] Wendel A. Glutathione peroxidase. Methods Enzymol 1981;77:325–332.

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

[34] Touma EH, Charpentier C. Medium chain acyl-CoA dehydrogenase deficiency. Arch Dis Child 1992;67:142–145.

[35] Iafolla AK, Thompson RJ Jr, Roe CR. Medium-chain acyl-coenzyme A dehydrogenase deficiency: clinical course in 120 affected children. J Pediatr 1994;124:409–415.

[36] Wilcken B, Hammond J, Silink M. Morbidity and mortality in medium chain acyl coenzyme A dehydrogenase deficiency. Arch Dis Child 1994;70:410–412.

[37] Pollitt RJ, Leonard JV. Prospective surveillance study of medium chain acyl-CoA dehydrogenase deficiency in the UK. Arch Dis Child 1998;79:116–119.

[38] Perper JA, Ahdab-Barmada M. Fatty liver, encephalopathy, and sudden unexpected death in early childhood due to medium-chain acyl-coenzyme A dehydrogenase deficiency. Am J Forensic Med Pathol 1992;13:329–334.

[39] Smith ET Jr, Davis GJ. Medium-chain acylcoenzyme-A dehydrogenase deficiency. Not just another Reye syndrome. Am J Forensic Med Pathol 1993;14:313–318.

[40] Mayatepek E, Koch HG, Hoffmann GF. Hyperuricaemia and medium-chain acyl-CoA dehydrogenase deficiency. J Inherit Metab Dis 1997;20:842–843.

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

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

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

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

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

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

[47] Latini A, Scussiato K, Rosa RB, Leipnitz G, Llesuy S, Bello-Klein A, Dutra-Filho CS, Wajner M. Induction of oxidative stress by L-2-hydroxyglutaric acid in rat brain. J Neurosci Res 2003;74:103–110.

[48] Latini A, Scussiato K, Rosa RB, Llesuy S, Bello-Klein A, Dutra-Filho CS, Wajner M. D-2-hydroxyglutaric acid induces oxidative stress in cerebral cortex of young rats. Eur J Neurosci 2003;17:2017–2022.

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

[50] de Oliveira Marques F, Hagen ME, Pederzolli CD, Sgaravatti AM, Durigon K, Testa CG, Wannmacher CM, de Souza Wyse AT, Wajner M, Dutra-Filho CS. Glutaric acid induces oxidative stress in brain of young rats. Brain Res 2003;964:153–158.

[51] Barschak AG, Sitta A, Deon M, de Oliveira Marques MH, Haeser A, Dutra-Filho CS, Wajner M, Vargas CR. Evidence that oxidative stress is increased in plasma from patients with maple syrup urine disease. Metab Brain Dis 2006;21:279–286.

[52] Sgaravatti AM, Sgarbi MB, Testa CG, Durigon K, Pederzolli CD, Prestes CG, Wyse AT, Wannmacher CM, Wajner M, Dutra-Filho CS. γ-Hydroxybutyric acid induces oxidative stress in cerebral cortex of young rats. Neurochem Int 2007;50:564–570.

[53] Halliwell B, Gutteridge JMC. Detection of free radicals and others reactive species: trapping and fingerprinting. In: Halliwell B, Gutteridge JMC, editors. Free radicals in biology and medicine. 4th ed. Oxford: Oxford University Press; 1999. p 351–425.

[54] Schönfeld P, Wojtczak L. Fatty acids decrease mitochondrial generation of reactive oxygen species at the reverse electron transport but increase it at the forward transport. Biochem Biophys Acta 2007;1767:1032–1040.

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

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

[57] Keston AS, Brandt R. The fluorometric analysis of ultramicro quantities of hydrogen peroxide. Anal Biochem 1965;11:1–5.

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

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

[60] Reiter RJ, Tan DX, Manchester LC, Qi W. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. Cell Biochem Biophys 2001;34:237–256.

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

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

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

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

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

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

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

[68] Hoffmann GF, Seppel CK, Holmes B, Mitchell L, Christen HJ, Hanefeld F, Rating D, Nyhan WL. Quantitative organic acid analysis in cerebrospinal fluid and plasma: reference values in a pediatric population. J Chromatogr 1993;617:1–10.

Capítulo IV

Medium-chain fatty acids accumulating in MCAD deficiency elicit lipid and protein oxidative damage and decrease non-enzymatic antioxidant defenses in rat brain

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Medium-chain fatty acids accumulating in MCAD deficiency elicit lipid and protein oxidative damage and decrease non-enzymatic antioxidant defenses in rat brain

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Running Title: Medium-chain fatty acids and oxidative stress

Medium-chain acyl-CoA dehydrogenase deficiency (MCADD) is the most frequent disorder of fatty acid oxidation with a similar prevalence to that of phenylketonuria. Affected patients present tissue accumulation of the medium-chain fatty acids octanoate (OA), decanoate (DA) and *cis*-4-decenoate. Clinical presentation is characterized by neurological symptoms, such as convulsions and lethargy that may develop into coma and sudden death. The aim of the present work was to investigate the *in vitro* effect of OA and DA, the metabolites that predominantly accumulate in MCADD, on oxidative stress parameters in rat cerebral cortex homogenates. It was first verified that both DA and OA significantly increased chemiluminescence and thiobarbituric acid-reactive species levels (lipoperoxidation) and decreased the non-enzymatic antioxidant defenses, measured by the decreased total antioxidant capacity. DA also enhanced carbonyl content and oxidation of sulfhydryl groups (protein damage) and decreased reduced glutathione (GSH) levels. We also verified that DA-induced GSH decrease and sulfhydryl oxidation were not observed when cytosolic preparations (membrane free-supenatants) were used, suggesting a mitochondrial mechanism for these actions. Our present data show that the medium-chain fatty acids DA and OA that most accumulate in MCADD cause oxidative stress in rat brain. It is therefore presumed that this pathomechanism may be involved in the pathophysiology of the neurologic symptoms manifested by patients affected by MCADD.

Keywords: **Reactive oxygen species; decanoic acid; octanoic acid; MCAD deficiency; lipid peroxidation; protein oxidation; oxidative stress; rat**

Introduction

The medium-chain fatty acids (MCFA) octanoate (OA), decanoate (DA) and *cis*-4-decenoate (cDA) accumulate in medium-chain acyl-coenzyme A dehydrogenase (MCAD, E.C. 1.3.99.3) deficiency (MCADD). This enzyme is responsible for the first step in mitochondrial β-oxidation of fatty acids, a process which provides energy, especially during fasting conditions (Roe and Ding, 2001). MCADD has been recognized as the most frequent defect of fatty acid oxidation (FAO), and its overall frequency is estimated to range between 1:6,500 to 1:17,000 newborns (Rinaldo et al., 2002). This disease is frequently diagnosed in children who present hypoketotic hypoglycemia, vomiting, lethargy, encephalopathy and seizures triggered by a common illness, which may rapidly progress to coma and death. However, once the diagnosis is established the prognosis is excellent, especially when detected before the onset of the symptoms, and frequent feedings are instituted to avoid prolonged period of fasting characterized by lipolysis and accumulation of the MCFA (Rinaldo et al., 2006).

At present, the pathogenesis of the wide range of metabolic disturbances in MCAD deficiency is poorly understood. Previous works from our group demonstrated that octanoic OA, DA and cDA acids, metabolites that accumulate in MCADD, were able to impair several parameters of energy metabolism in cerebral cortex of rats, including the respiratory chain complexes, mitochondrial creatine kinase and Na^+, K^+-ATPase activities (de Assis et al., 2003, 2006; Reis de Assis et al., 2004), enzymes that are higly susceptible to oxidative damage (Lees, 1993; Wolosker et al., 1996; Kurella et al., 1997; Arstall et al., 1998; Stachowiak et al., 1998; Yousef et al., 2002). It was recently demonstrated that cDA provokes alterations in various parameters of oxidative stress in brain of young rats (Schuck et al., 2007) and a study has shown that OA and DA induce lipid peroxidation in

cerebral cortex of young rats (de Assis et al., 2006). Therefore, considering that OA and DA are the major metabolites accumulating in MCADD, in the present work we investigated in more details the *in vitro* effects of these MCFA on a wide spectrum of oxidative stress parameters, namely thiobarbituric acid-reactive substances (TBA-RS), spontaneous chemiluminescence, carbonyl content, sulfhydryl groups oxidation, total-radical trapping antioxidant potential (TRAP), reduced glutathione (GSH) levels and nitrates and nitrites production in homogenates from cerebral cortex of young rats in the hope to contribute for a better understanding of the mechanisms underlying the neurological dysfunction found in MCAD-deficient patients.

Experimental Procedure

Reagents

All chemicals were purchased from Sigma (St. Louis, MO, USA). OA and DA were dissolved on the day of the experiments in the incubation medium used for each technique with pH adjusted to 7.4. The final concentrations of these fatty acids in the medium ranged from 0.5 to 3.0 mM.

Animals

Thirty-day-old male Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, were used. Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned

constant temperature (22±1°C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

Tissue preparation and incubation

On the day of the experiments the animals were sacrificed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum and striatum were discarded, and the cerebral cortex was peeled away from the subcortical structures, weighed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 x *g* for 10 min at 4ºC to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl at 37° C for one hour with OA or DA at concentrations of 0.5, 1 or 3 mM. Controls did not contain any of these metabolites in the incubation medium. Immediately after incubation, aliquots were taken to measure the values of TRAP, GSH concentrations, TBA-RS levels, spontaneous chemiluminescence, nitrates and nitrites levels and carbonyl and sulfhydryl content. For some experiments, the supernatant was separated and centrifuged at 100,000 x *g* for one hour and the final supernatant, free of membranes, representing a cellular cytosolic preparation, was incubated in the same buffer at 37° C for one hour with DA at

concentration of 3 mM. After this incubation, aliquots were taken to measure the values of GSH levels and sulfhydryl content.

We always carried out parallel experiments with various blanks (controls) in the presence or absence of the tested metabolites (OA and DA) and also with or without cortical supernatants in order to detect artifacts caused by the fatty acids in the assays. By doing so, any interference of these acids on the reactions used to measure the oxidative stress parameters would be identified.

Thiobarbituric acid-reactive substances (TBA-RS) levels

TBA-RS was determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10 % trichloroacetic acid were added to 150 μL of OA or DA pre-treated cerebral cortex supernatants and centrifuged at 300 x *g* for 10 min. Three hundred microliters of the supernatant were transferred to a pyrex tube and incubated with 300 μL of 0.67 % TBA in 7.1 % sodium sulphate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for five min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. Some experiments were performed in the presence or absence of reduced glutathione (GSH; 100 μM), melatonin (MEL; 100 μM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 500 μM), trolox (TRO; 5 μM) or a combination of catalase (CAT; 10 mU . mL^{-1}) plus superoxide dismutase (SOD; 10 mU . mL^{-1}). TBA-RS values were calculated as nmol of TBA-RS. mg $protein^{-1}$.

Spontaneous Chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room by the method of Gonzalez Flecha et al. (1991). Incubation flasks contained 3.5 mL of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl were counted for background chemiluminescence during five minutes. An aliquot of 500 μL of cortical supernatant was immediately added and the spontaneous chemiluminescence was measured for 30 min at room temperature. The background chemiluminescence was subtracted from the total value and the results were calculated as cpm . mg protein^{-1}.

Determination of protein carbonyl formation content

Protein carbonyl content formation, a marker of oxidized proteins, was measured spectrophotometrically according to Reznick and Packer (1994). One hundred microliters of the aliquots from the incubation were treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank control) and left in the dark for one hour. Samples were then precipitated with 500 μL 20 % TCA and centrifuged for five min at 10,000 x *g*. The pellet was then washed with 1 mL ethanol: ethyl acetate (1:1, v/v) and re-dissolved in 550 μL 6 M guanidine prepared in 2.5 N HCl Then, the tubes were incubated at 37 º C for five min to assure the complete dissolution of the pellet and the resulting sample was determined at 365 nm. The difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl content. The results were calculated as nmol of carbonyls groups . mg of protein^{-1}, using the extinction coefficient of 22,000 x 106 nmol . mL^{-1} for aliphatic hydrazones.

Total radical-trapping antioxidant potential (TRAP)

TRAP, representing the total non-enzymatic antioxidant capacity of the tissue, was determined by measuring the chemiluminescence intensity of luminol induced by 2,2'-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. (1992). The reaction mixture containing 4 mL 10 mM ABAP dissolved in 0.1 M glycine buffer, pH 8.6 and 10 µL of luminol (4 mM) generates, at room temperature, an almost constant light intensity corresponding to free radical formation, which was measured in a Wallac 1409 liquid scintillation counter. This was considered to be the initial chemiluminescence values. Then, 30 µL of 300 µM trolox (soluble α-tocopherol analogue) or 30 µL of cortical supernatants were added to the reaction medium. The addition of trolox or supernatants provokes a marked reduction of the light intensity, which is maintained for a certain period after which light intensity rapidly increase. This period corresponds to induction time (IT) and represents TRAP measurement. IT is directly proportional to the antioxidant capacity of the tissue, and the IT of each sample was compared with the IT of trolox. TRAP values were expressed as nmol trolox . mg of protein^{-1}.

Reduced glutathione (GSH) concentrations

Reduced glutathione (GSH) concentrations were measured according to Browne and Armstrong (1998). OA and DA pre-treated cerebral cortex supernatants were diluted in 20 volumes of (1:20, v/v) 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation were incubated with an equal volume

of *o*-phthaldialdehyde (1 mg . mL methanol^{-1}) at room temperature during 15 minutes. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Some experiments were performed in the presence or absence of melatonin (MEL; 100 μM), trolox (TRO; 5 μM) or catalase (CAT; 10 mU . mL^{-1}) plus superoxide dismutase (SOD; 10 mU . mL^{-1}). Calibration curve was prepared with standard GSH (0.01 - 1 mM) and the concentrations were calculated as nmol . mg protein^{-1}.

The oxidation of a commercial solution of GSH (200 μM) was also tested by exposing this solution to 0.5 - 3 mM OA or DA for one hour in a medium devoid of brain supernatants. After OA and DA exposition, 7.4 mM *o*-phthaldialdehyde was added to the vials and the mixture was incubated at room temperature during 15 min.

Nitrate and nitrite determination

Nitrate and nitrite concentrations were determined according to Miranda et al., 2001. Briefly, 12 μL of 20 % trichloroacetic acid were added to 300 μL of OA or DA pre-treated cerebral cortex supernatants and centrifuged at 12,000 x *g* for 10 min. Two hundred microliters of the supernatant were transferred to a eppendorf tube and incubated with 200 μL of 0.8 % VCl_3 in 1 M HCl and 200μL of the Griess reagent (2% sulfanilamide in 5% HCl and 0.1% *N*-1-(naphtyl)ethylenediamine in H_2O) at 37ºC for 30 min in a dark room. Absorvance was then determined at 540 nm by spectrophotometry. A calibration curve was performed using sodium nitrate, and each curve point was subjected to the same treatment as supernatants and the concentrations were calculated as μmol . mg protein^{-1}.

Protein determination

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

Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. Linear regression analysis was used to detect dose-dependent effects . Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Results

OA and DA induce lipid peroxidation

We initially tested the influence of OA and DA on the lipid peroxidation parameters TBA-RS levels and chemiluminescence. Figure 1 shows that when cortical homogenates were exposed for 60 min to DA and OA, at concentrations of 0.5 mM and higher, TBA-RS levels significantly increased up to 147% [$F_{(3,23)}$=40.7; $P < 0.001$] and 36% [$F_{(3,23)}$=8.18; $P < 0.001$], respectively. Moreover, DA increased TBA-RS levels in a dose-dependent manner [β=0.91; $P < 0.001$]. Similar results were obtained for spontaneous chemiluminescence,in which both fatty acids significantly increased this parameter up to

approximately 38% [DA: $F_{(3,19)}$=3.26; $P < 0.05$; OA: $F_{(3,20)}$=3.35; $P < 0.05$], but only at 1 mM and higher concentrations.

["Figure 1 about here."]

Next, we incubated cerebral cortex homogenates during 15, 30, 45 or 60 min in the presence or absence of OA or DA (0.5-3.0 mM) and observed a significant stimulatory effect of 1.0 and 3.0 mM OA on lipid peroxidation (TBA-RS) only after 60 min incubation. On the other hand, DA was already able to induce lipid peroxidation after 30 min incubation at 3 mM concentration and after 45 min at 0.5 mM and higher doses (Figure 2).

["Figure 2 about here."]

We also tested the effect of the antioxidants reduced glutathione (GSH; 100 μM), melatonin (MEL; 100 μM), the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 500 μM), trolox (TRO; 5 μM), catalase (CAT; 10 mU . mL^{-1}) plus superoxide dismutase (SOD; 10 mU . mL^{-1}) on the DA-elicited increase of TBA-RS levels. These antioxidants were co-incubated with 3 mM DA and brain cortical homogenates for 60 min, after which the levels of TBA-RS were measured. We observed that MEL and TRO fully prevented DA-induced increased lipid peroxidation, whereas GSH partially prevented such effect [$F_{(6,34)}$=25.8; $P < 0.01$] (Figure 3). In contrast, CAT plus SOD and L-NAME were not able to prevent the increase of TBA-RS values caused by DA.

["Figure 3 about here."]

DA induces protein oxidative damage in rat cerebral cortex

We then evaluated the effect of OA and DA on carbonyl formation and sulfhydryl oxidation in cortical homogenates in order to evaluate protein oxidation parameters. We found that DA significantly increased protein carbonyl groups content (up to 340 %) in cortical homogenates [$F_{(3,15)}$=25.9; $P < 0.001$] in a dose-dependent manner [β= 0.89; $P < 0.001$] (Figure 4A) and induced sulfhydryl oxidation (up to 61 %) at 3 mM [$F_{(3,19)}$=15.5; $P < 0.001$] (Figure 4B). In contrast, OA did not affect any of these parameters.

["Figure 4 about here."]

Non-enzymatic antioxidant defenses are decreased by OA and DA in rat cerebral cortex

We also investigated the *in vitro* effect of OA and DA on the non-enzymatic antioxidant defenses, by measuring TRAP and GSH levels. We found that both fatty acids significantly decreased TRAP values (OA up to 20 % and DA up to 57 %) in rat cortical homogenates (OA: [$F_{(3,19)}$=3.90; $P < 0.05$]; DA: [$F_{(3,15)}$=22.9; $P < 0.001$]), and DA-induced effect was in a dose-dependent manner [β= 0.84; $P < 0.001$] (Figure 5A). On the other hand, only DA diminished GSH levels [$F_{(3,19)}$=9.09; $P < 0.001$] in a dose-dependent manner [β= 0.78; $P < 0.001$] (Figura 5B). Furthermore, this effect was totally prevented by the free-radical scavengers MEL and TRO, but not by CAT plus SOD [$F_{(4,19)}$=13.3; $P < 0.001$] (Figura 5C). Taken together, the data indicate a marked reduction of the brain non-enzymatic antioxidant defenses by DA and to a lesser extend by OA. Furthermore, DA-induced reduction of TRAP (total tissue non-enzymatic antioxidant defenses) was inversely correlated with TBA-RS values (a lipid peroxidation parameter) [β= -0.83; $P < 0.001$], suggesting that free radicals inducing lipid peroxidation were probably responsible for the decrease of the non-enzymatic antioxidant defenses caused by this fatty acid (Figure 6).

["Figures 5 and 6 about here."]

DA does not directly react with a commercial solution of GSH

We then investigated whether the DA-induced GSH levels decrease was due to a direct oxidative attack. We therefore exposed a commercial solution of GSH (200 μM) to 0.5-3.0 mM DA for 60 min in the absence of tissue supernatants. It was observed that DA did not oxidize free GSH, suggesting that this medium chain fatty acid *per se* is not a direct oxidant agent, but elicits oxidative damage probably via free radical generation (Table I).

["Table I about here."]

OA and DA did not affect nitrite and nitrate concentrations in rat cerebral cortex

We next evaluated the effects of OA and DA on nitrate and nitrite production, in order to verify if these acids could induce reactive nitrogen species (RNS) generation. We observed that RNS production was not altered by OA and DA (data not shown).

DA does not alter GSH levels and sulfhydryl content when incubated with cytosolic preparations

In these experiments, we exposed cytosolic preparations (membrane free-supernatants) from cerebral cortex to 3 mM DA for 60 min and afterwards determined GSH levels and sulfhydryl content. We observed that DA did not cause any effect on these parameters (GSH levels: [$t_{(5)}$=0.16; $P > 0.05$]; SH content: [$t_{(5)}$=-0.64; $P > 0.05$]), probably signalizing the role of mitochondrial mechanisms for DA-induced effects (Figure 7).

["Figure 7 about here."]

Discussion

A considerable number of patients with MCADD present a variable degree of neurological dysfunction associated with cerebral MRI alterations (Perper and Ahdab-Barmada, 1992; Smith and Davis, 1993; Ruitenbeek et al., 1995; Mayatepek et al., 1997; Wilson et al., 1999; Scrace et al., 2008) whose pathophysiology is not yet clear. Lethargy that may progress to coma and death is believed to be particularly due to the accumulation of toxic MCFA and/or their by-products (Gregersen et al., 2008). It may be therefore presumed that the increased tissue concentrations of OA and DA, the most pronounced MCFA accumulating in this disorder, contribute to the neurological dysfunction of the affected patients. In this context, OA and DA were shown to provoke alterations of bioenergetics and induce oxidation of lipids in rat cerebral cortex (de Assis et al., 2006), whereas cDA to induce oxidative damage and reduce the antioxidant defenses in brain from young rats (Schuck et al., 2007).

Therefore, considering that oxidative stress is considered an important pathomechanism of common neurodegenerative disorders (Perez-Severiano et al., 2000; Bogdanov et al., 2001; Behl and Moosmann, 2002; Stoy et al., 2005; Berg and Youdim, 2006; Mancuso et al., 2006) and of some inborn errors of metabolism (Latini et al., 2003a, 2003b, 2005; de Oliveira Marques et al., 2003; Barschaket al., 2006; Sgaravatti et al., 2007) and that the role of OA and DA on biological oxidations was not yet fully evaluated, the present work investigated the *in vitro* influence of these MCFA on a large number of oxidative stress parameters reflecting oxidative damage and the antioxidant defenses content in cerebral cortex of young rats.

We first observed that OA and DA markedly increased TBA-RS and spontaneous chemiluminescence at concentrations of 0.5 mM and higher and that DA effects were more pronounced. Since light emitted in the spontaneous chemiluminescence assay usually arises from peroxidizing lipids due to an increase in reactive oxygen or nitrogen species production and TBA-RS reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge, 1999), it may be concluded that these fatty acids induce lipid peroxidation. These results confirm previous data from our laboratory (de Assis et al., 2006). Furthermore, TBA-RS values gradually increased when cerebral cortex homogenates were exposed for increasing periods to OA and DA, suggesting that free radical synthesis was probably involved in these effects.

We also found that the strong *in vitro* lipid peroxidation induced by DA was totally prevented by the reactive species scavengers TRO and MEL and attenuated by GSH. In contrast, L-NAME or a combination of the antioxidant enzymes CAT plus SOD were not able to change DA-elicited increase of TBA-RS levels. These data reinforce the presumption that the lipid oxidative effect exerted by DA was probably mediated by reactive oxygen species generation. Considering that TRO is effective against peroxyl and alkoxyl radicals (Halliwell and Gutteridge, 2007a) and MEL scavenges mainly the reactive species hydroxyl, peroxyl and nitric oxide/peroxynitrite anion (Maharaj et al., 2007), whereas the nitric oxide inhibitor L-NAME and CAT plus SOD were unable to avoid peroxidation of lipids caused by DA, it may be presumed that peroxyl, alkoxyl and hydroxyl radicals were mainly involved in DA action. We cannot, however, rule out the involvement of other mechanisms leading to oxidative damage since MEL has other antioxidant properties including the activation of antioxidant enzymes (Maharaj et al., 2007). In this context, it may be also presumed that DA also provoked oxidation of

sulfhydryl groups since GSH attenuated its effects on lipid peroxidation and is considered an excellent antioxidant agent protecting thiol groups from oxidation, besides being able to react with a wide spectrum of reactive species (Halliwell and Gutteridge, 2007b). Overall, the data indicate that DA and OA provoked a strong pro-oxidant effect on membrane lipids from cerebral cortex probably mediated by reactive oxygen species generation.

Protein carbonyl formation and sulfhydryl oxidation were also significantly increased by DA, but not by OA, indicating that these MCFA act through different mechanisms. Since carbonyl formation is a marker for assessing oxidative protein damage and most cellular sulfhydryl groups are protein-bound, it is conceivable that DA also provokes protein oxidation (Reznick and Packer, 1994; Levine, 2002). On the other hand, considering that DA was not able to oxidize a commercial reduced glutathione in the absence of brain supernatants in the incubation medium, it could be presumed that this fatty acid is not *per se* a direct oxidant agent and that it probably provoked lipid and protein oxidative damage via free radical induction.

Regarding to the antioxidant defenses, OA and DA reduced the total content of non-enzymatic antioxidants (TRAP values), but only DA decreased GSH levels, the main naturally-occurring brain antioxidant, in cerebral cortex. Therefore, it is possible that OA acted on other brain antioxidant defenses, rather than on GSH. Since these parameters are used to evaluate the capacity of a tissue to prevent and respond to damage associated to free radical processes (Lissi et al., 1995; Halliwell and Gutteridge, 1999; Evelson et al., 2001), it may be concluded that the rat cortical antioxidant defenses were severely compromised by DA, and to a lesser extend by OA. Moreover, we verified that MEL and TRO, but not SOD plus CAT, were able to prevent DA-decrease of GSH levels in cortical supernatants,

corroborating the idea that peroxyl, alkoxyl and hydroxyl radicals were mainly involved in the reduction of GSH provoked by DA.

Since adequate levels of antioxidants are essential to protect cells against oxidative damage and an imbalance in the pro-oxidant/antioxidant homeostasis induces oxidative stress, it is possible that the significant reduction of GSH induced by DA may be related at least in part to the increased *in vitro* lipid peroxidation caused by this antioxidant. This is in line with the observations that GSH is considered an important defense against lipid oxidative damage eliminating hydrogen peroxide and peroxyl radicals formed during this process and that supplementation of GSH to cortical supernatants attenuated *in vitro* lipid oxidative damage induced by DA in cerebral cortex. On the other hand, we cannot rule out that GSH decrease could be secondary to the increased reactive species generation provoked by DA. In this context, we found a strong inverse correlation between TBA-RS increase and TRAP decrease caused by DA, indicating that the decrease of the brain antioxidant defenses and the induction of lipid peroxidation are dependent effects. Therefore, it is feasible that the marked decline of the cerebral non-enzymatic antioxidant defenses (TRAP and GSH) probably reflects the rapid consume of highly reactive antioxidants due to the generation of free radicals elicited by DA.

Now, regarding the mechanisms by which DA exerted its effects, we observed that this fatty acid at a high concentration (3 mM) was unable to change GSH levels and sulfhydryl content when incubated with cytosolic preparations purified from cerebral cortex, which are devoid of mitochondria. These results allied to the fact that DA markedly reduced GSH levels and sulfhydryl content when supplemented to whole homogenates that contain all cell machinery, and considering that mitochondria is the main source of reactive

oxygen species (Halliwell and Gutteridge, 2007c), it is feasible that mitochondria exerted an important role in DA-induced effects.

Since oxidative stress results from an imbalance between the total tissue antioxidant defense and the reactive species generated, our present data strongly indicate that OA and particularly DA provoke a significant *in vitro* stimulation of oxidative stress in cerebral cortex, a deleterious cell condition that induces oxidation of lipids and proteins and reduces tissue antioxidant defenses (Halliwell and Gutteridge, 1999). At this point, it should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues (Halliwell and Gutteridge, 1996), a fact that makes this tissue more vulnerable to increased reactive species.

At the present we cannot establish whether our data have a pathophysiological significance since brain concentrations of the MCFA in MCAD-deficient patients are unknown. It should be however noted that the significant alterations of the oxidative stress parameters elicited by OA and DA occurred at 0.5 mM and higher doses, which are similar that those found in blood and CSF of the affected patients particularly during metabolic crises that are characterized by acute encephalopathy and by a dramatic increase in the concentrations of the accumulating metabolites (Martínez et al., 1997; Roe and Ding, 2001). In case the present findings are confirmed *in vivo* in animal experiments and also in tissues of patients affected by MCADD, it is tempting to speculate that excessive reactive species generation might contribute, at least in part, to the neuropathology of this disorder. It seems therefore reasonable to propose that antioxidants may serve as an adjuvant therapy to specific diets or to other pharmacological agents used for these patients especially during crises to avoid oxidative damage to the brain.

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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. Journal of Molecular and Cellular Cardiology 30, 979–988.

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

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

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

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

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

de Assis, D. R., Maria, R. C., Ferreira, G. C., Schuck, P. F., Latini, A., Dutra-Filho, C. S., Wannmacher, C. M., Wyse, A. T., Wajner, M., 2006. Na^+, K^+ ATPase activity is markedly reduced by *cis*-4-decenoic acid in synaptic plasma membranes from cerebral cortex of rats. Experimental Neurology 197, 143-149.

de Assis, D. R., Ribeiro, C. A., Rosa, R. B., Schuck, P. F., Dalcin, K. B., Vargas, C. R., Wannmacher, C. M., Dutra-Filho, C. S., Wyse, A. T., Briones, P., Wajner, M., 2003. Evidence that antioxidants prevent the inhibition of Na^+,K^+-ATPase activity induced by octanoic acid in rat cerebral cortex *in vitro*. Neurochemical Research 28, 1255-1263.

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

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

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

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

Gregersen, N., Andresen, B. S., Pedersen, C. B., Olsen, R. K., Corydon, T. J., Bross, P., 2008. Mitochondrial fatty acid oxidation defects-remaining challenges. J. Inherit. Metab. Dis. *In press.*

Halliwell, B., Gutteridge, J. M. C., 1996. Oxygen radicals and nervous system. Trends in Neuroscience 8, 22–26.

Halliwell, B., Gutteridge, J. M. C., 1999. Detection of free radicals and others reactive species: trapping and fingerprinting. In: Halliwell, B., Gutteridge, J. M. C. (Ed.) Free Radicals in Biology and Medicine. Oxford University Press, Oxford, pp. 351–425.

Halliwell, B., Gutteridge, J. M. C., 2007a. Ageing, nutrition, disease and therapy: a role for antioxidants? In: Halliwell, B., Gutteridge, J. M. .C. (Ed.) Free Radicals in Biology and Medicine. Oxford University Press, Oxford, pp. 614-677.

Halliwell, B., Gutteridge, J. M. C., 2007b. Antioxidant defences: endogenous and diet derived. In: Halliwell, B., Gutteridge, J. M. .C. (Ed.) Free Radicals in Biology and Medicine. Oxford University Press, Oxford, pp. 79-186.

Halliwell, B., Gutteridge, J. M. C., 2007c. Oxygen is a toxic gas – an introduction to oxygen toxicity and reactive species. In: Halliwell, B., Gutteridge, J. M. .C. (Ed.) Free Radicals in Biology and Medicine. Oxford University Press, Oxford, pp. 1-29.

Kurella, E., Kukley, M., Tyulina, O., Dobrota, D., Matejovicova, M., Mezesova, V., Boldyrev, A., 1997. Kinetic parameters of Na/K-ATPase modified by free radicals *in vitro* and *in vivo*. Annals of the New York Academy of Science 834, 661–665.

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

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

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

Lees, G. J., 1993. Contributory mechanisms in the causation of neurodegenerative disorders. Neuroscience 54, 287–322.

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

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

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

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

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

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

Martínez, G., Jiménez-Sánchez, G., Divry, P., Vianey-Saban, C., Riudor, E., Rodés, M., Briones, P., Ribes, A., 1997. Plasma free fatty acids in mitochondrial fatty acid oxidation defects. Clinica Chimica Acta 267, 143-154.

Mayatepek, E., Koch, H. G., Hoffmann, G. F., 1997. Hyperuricaemia and medium-chain acyl-CoA dehydrogenase deficiency. Journal of Inherited Metabolic Disease 20, 842-843.

Miranda, K. M., Espey, M. G., Wink, D. A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide: Biology and Chemistry 1, 62-71.

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

Perper, J. A., Ahdab-Barmada, M., 1992. Fatty liver, encephalopathy, and sudden unexpected death in early childhood due to medium-chain acyl-coenzyme A dehydrogenase deficiency. The American Journal of Forensic Medicine and Pathology 13, 329-334.

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

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

Rinaldo, P., Hahn, S., Matern, D., 2006. Inborn errors of amino acid, organic acid, and fatty acid metabolism. In: Burtis, C. A., Ashwood, E. R., Bruns, D. E. (Ed.) Tietz textbook of clinical chemistry and molecular diagnostics. 4th Edition. Elsevier Saunders, St. Louis, pp. 2207-2247.

Rinaldo, P., Matern, D., Bennett, M. J., 2002. Fatty acid oxidation disorders. Annual Review of Physiology 64, 477-502.

Roe, C. R., Ding, J., 2001. Mitochondrial fatty acid oxidation disorders. In: Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D. (Ed.) The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 1909–1963.

Ruitenbeek, W., Poels, P. J., Turnbull, D. M., Garavaglia, B., Chalmers, R. A., Taylor, R. W., Gabreels, F. J., 1995. Rhabdomyolysis and acute encephalopathy in late onset medium chain acyl-CoA dehydrogenase deficiency. Journal of Neurology, Neurosurgery, and Psychiatry 58, 209-214.

Schuck, P. F., Ceolato, P. C., Ferreira, G. C., Tonin, A., Leipnitz, G., Dutra-Filho, C. S., Latini, A., Wajner, M., 2007. Oxidative stress induction by *cis*-4-decenoic acid: relevance for MCAD deficiency. Free Radical Research 41, 1261-1272.

Scrace, B., Wilson, P., Pappachan, J., 2008. Medium chain acyl CoA dehydrogenase deficiency causes unexplained coma in a 10-year-old child. Paediatr Anaesth, In press.

Sgaravatti, A. M., Sgarbi, M. B., Testa, C. G., Durigon, K., Pederzolli, C. D., Prestes, C. G., Wyse, A. T., Wannmacher, C. M., Wajner, M., Dutra-Filho, C. S., 2007. γ-

Hydroxybutyric acid induces oxidative stress in cerebral cortex of young rats. Neurochemistry International 50, 564-570.

Smith, E. T. Jr, Davis, G. J., 1993. Medium-chain acylcoenzyme-A dehydrogenase deficiency. Not just another Reye syndrome. The American Journal of Forensic Medicine and Pathology 14, 313-318.

Stachowiak, O., Dolder, M., Wallimann, T., Richter, C., 1998. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. The Journal of Biological Chemistry 273, 16694-16699.

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

Wilson, C. J., Champion, M. P., Collins, J. E., Clayton, P. T., Leonard, J. V., 1999. Outcome of medium chain acyl-CoA dehydrogenase deficiency after diagnosis. Archives of Disease in Childhood 80, 459-462.

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

Yousef, M. I., El-Hendy, H. A., El-Demerdash, F. M., Elagamy, E. I., 2002. Dietary zinc deficiency induced-changes in the activity of enzymes and the levels of free radicals, lipids and protein electrophoretic behavior in growing rats. Toxicology 175, 223–234.

Legends to Figures

Figure 1. *In vitro* effect of octanoic (OA) and decanoic (DA) acids on the lipid peroxidation parameters spontaneous chemiluminescence *(A)* and thiobarbituric acid-reactive substances (TBA-RS) *(B)* in rat cerebral cortex. Values are means ± standard deviation for six independent experiments performed in duplicate or triplicate. TBA-RS levels are expressed as nmol . mg protein^{-1} and chemiluminescence as cpm . mg protein^{-1}. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to controls (Duncan multiple range test).

Figure 2. *In vitro* effect of octanoic (OA) and decanoic (DA) acids on thiobarbituric acid-reactive substances (TBA-RS) in rat cerebral cortex exposed for different periods to the acids. Cortical homogenates were incubated for 15, 30, 45 or 60 min with OA or DA (0.5-3.0 mM) and TBA-RS levels measured afterwards. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as nmol . mg protein^{-1}. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to controls (Duncan multiple range test).

Figure 3. *In vitro* effect of the antioxidants GSH (100 μM), MEL (100 μM), L-NAME (500 μM), TRO (5 μM) and CAT plus SOD (10 mU . mL^{-1} each) on decanoic (DA)-induced increase of thiobarbituric-acid reactive substances (TBA-RS) in rat cerebral cortex. Cortical homogenates were pre-incubated for 15 min with the antioxidants before the addition of the 3 mM DA. Values are means ± standard deviation for four independent experiments performed in triplicate and are expressed as nmol . mg protein^{-1}. ** $P < 0.01$;

*** $P < 0.001$ compared to controls; #$P < 0.05$ compared to DA (Duncan multiple range test).

Figure 4. *In vitro* effect of octanoic (OA) and decanoic (DA) acids on carbonyl *(A)* and sulfhydryl *(B)* content in rat cerebral cortex. Values are means ± standard deviation for five independent experiments performed in triplicate and are expressed as nmol . mg protein^{-1}. *$P < 0.05$, ***$P < 0.001$ compared to control (Duncan multiple range test).

Figure 5. *In vitro* effect of octanoic (OA) and decanoic (DA) acids on the non-enzymatic antioxidant parameters total-radical trapping antioxidant potential (TRAP) *(A)* and reduced glutathione (GSH) levels *(B)* in rat cerebral cortex. The effects of DA on GSH levels in the presence of the antioxidants MEL (100 μM), TRO (5 μM) and CAT plus SOD (10 mU . mL^{-1} each) were also tested *(C)*. Values are means ± standard deviation for five to nine independent experiments performed in duplicate or triplicate and are expressed as nmol . mg protein^{-1}. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to controls (Duncan multiple range test).

Figure 6. Correlation between thiobarbituric acid-reactive substances (TBA-RS) and total-radical trapping antioxidant potential (TRAP) in rat cerebral cortex exposed to 0.5-3.0 mM decanoic (DA) acid. Values are means ± standard deviation for five to six independent experiments performed in triplicate and are expressed as nmol . mg protein^{-1} for TBA-RS and TRAP.

Figure 7. *In vitro* effect of decanoic acid (DA) on reduced glutathione (GSH) levels *(A)* and sulfhydryl content *(B)* in cytosolic preparations from rat cerebral cortex. Values are means ± standard deviation for five independent experiments performed in triplicate and are expressed as nmol . mg protein^{-1}.

Figure 1.

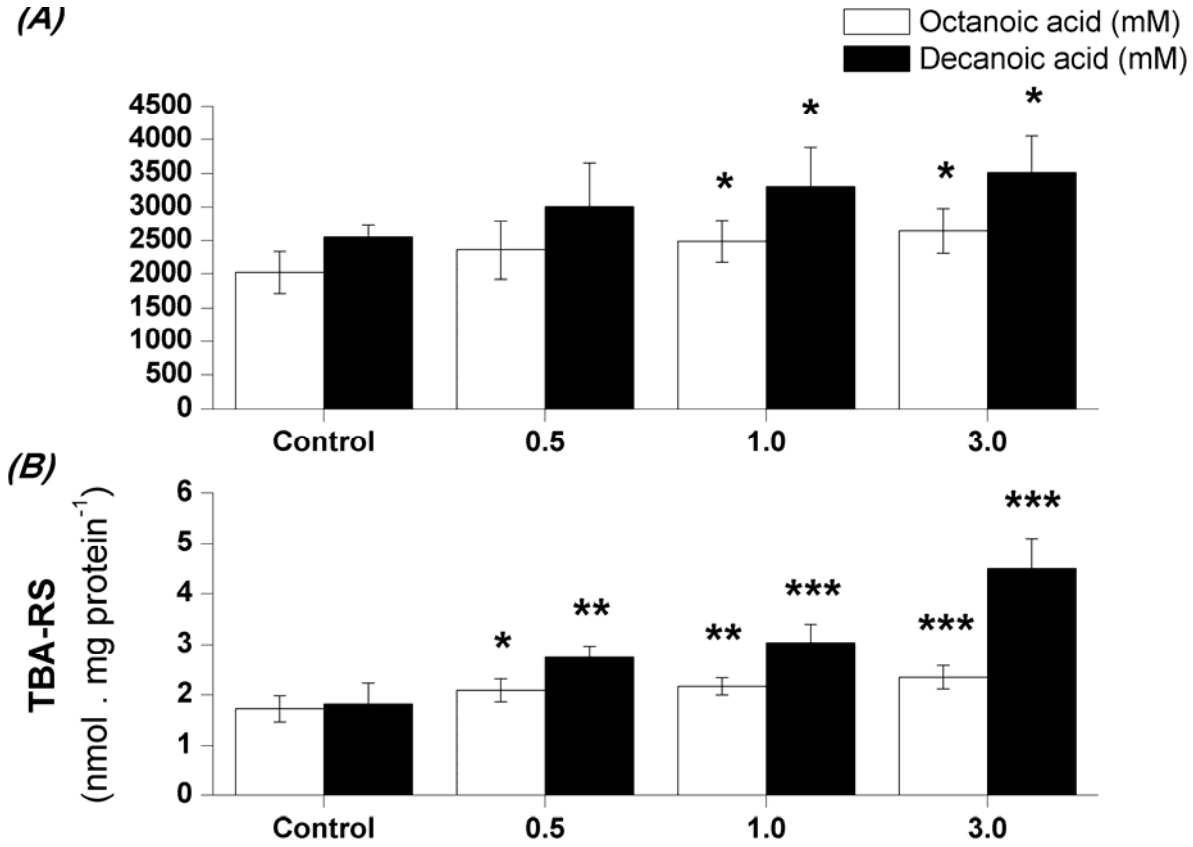

(A)
Octanoic acid (mM)
Decanoic acid (mM)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Control
0.5
1.0
3.0
(B)
TBA-RS
(nmol . mg protein-1)
6
5
4
3
2
1
0
Control
0.5
1.0
3.0

Figure 2.

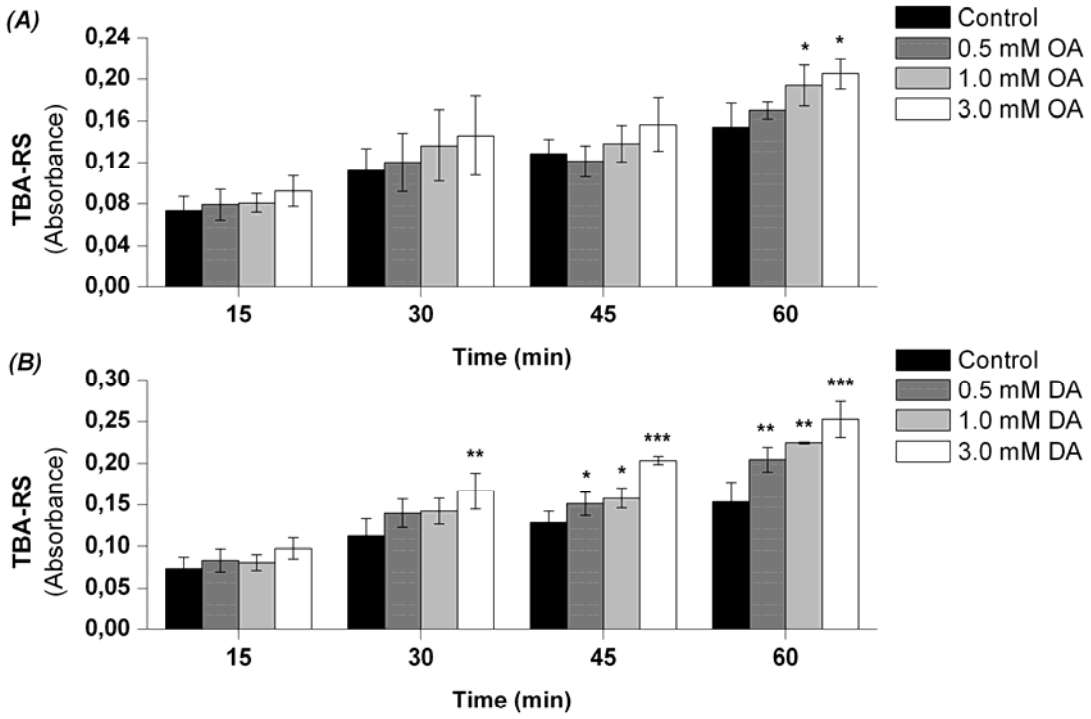

(A)
TBA-RS (Absorbance)
0,24
0,20
0,16
0,12
0,08
0,04
0,00
15
30
45
60
Time (min)
Control
0.5 mM OA
1.0 mM OA
3.0 mM OA
(B)
TBA-RS (Absorbance)
0,30
0,25
0,20
0,15
0,10
0,05
0,00
15
30
45
60
Time (min)
Control
0.5 mM DA
1.0 mM DA
3.0 mM DA

Figure 3.

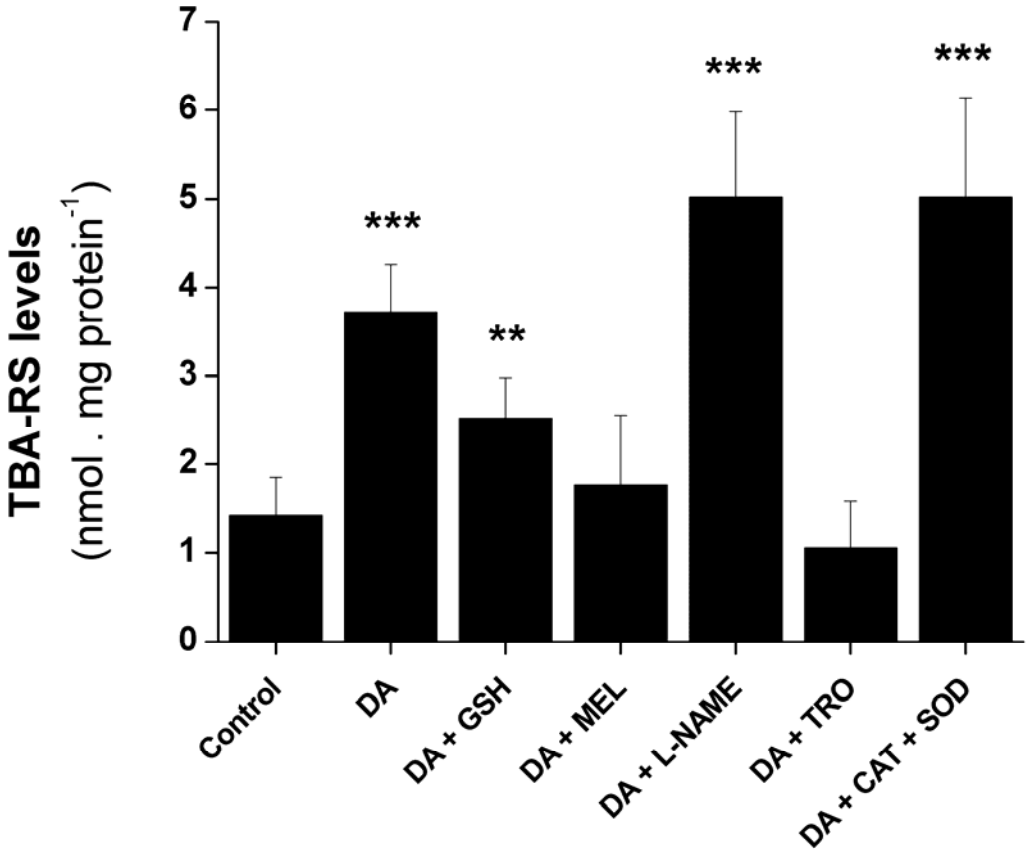

TBA-RS levels
(nmol . mg protein-1)
0
1
2
3
4
5
6
7
***
**
***
***
Control
DA
DA + GSH
DA + MEL
DA + L-NAME
DA + TRO
DA + CAT + SOD

Figure 4.

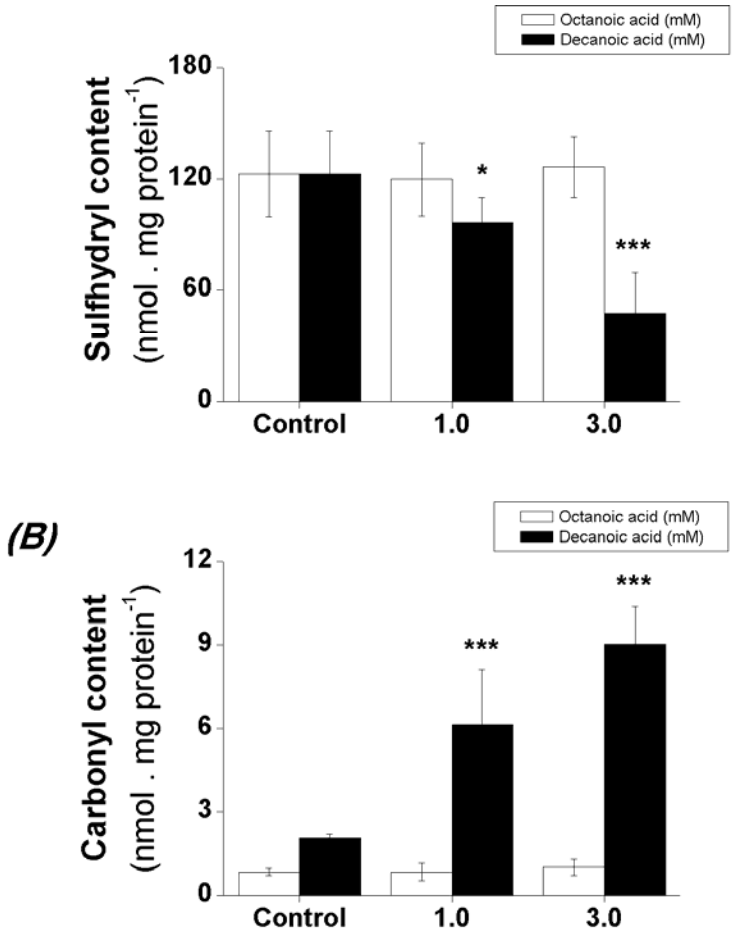

Octanoic acid (mM)
Decanoic acid (mM)
Sulfhydryl content
(nmol . mg protein-1)
180
120
60
0
*
***
Control
1.0
3.0
(B)
Octanoic acid (mM)
Decanoic acid (mM)
Carbonyl content
(nmol . mg protein-1)
12
9
6
3
0
***
***
Control
1.0
3.0

Figure 5.

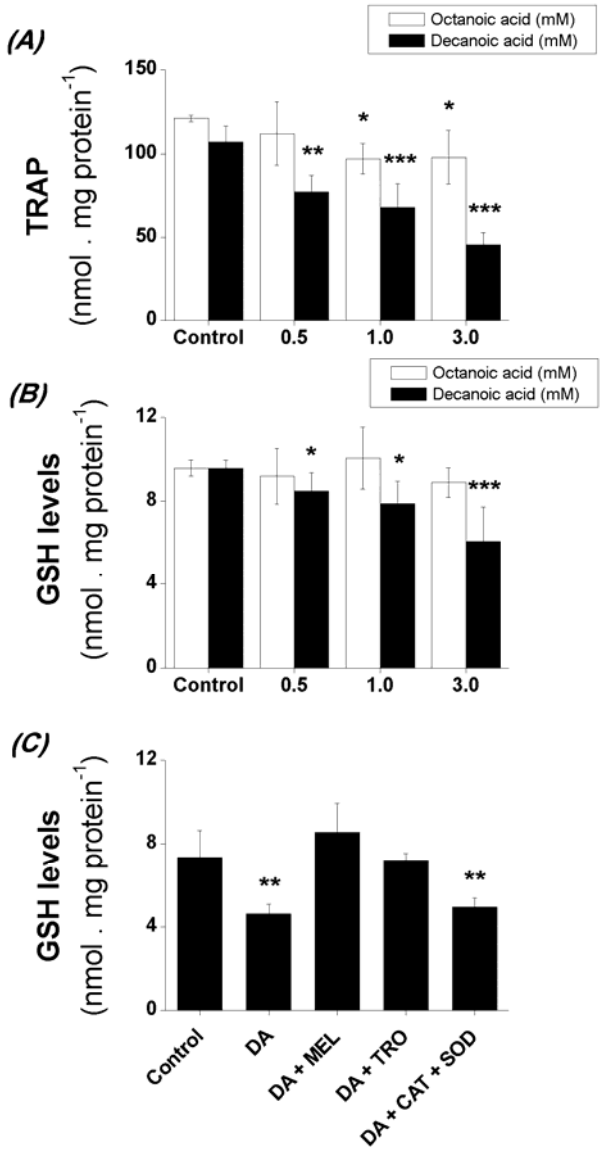

(A)
Octanoic acid (mM)
Decanoic acid (mM)
TRAP
(nmol . mg protein-1)
150
100
50
0
Control
0.5
1.0
3.0
(B)
GSH levels
(nmol . mg protein-1)
12
8
4
0
(C)
DA
DA + MEL
DA + TRO
DA + CAT + SOD

Figure 6.

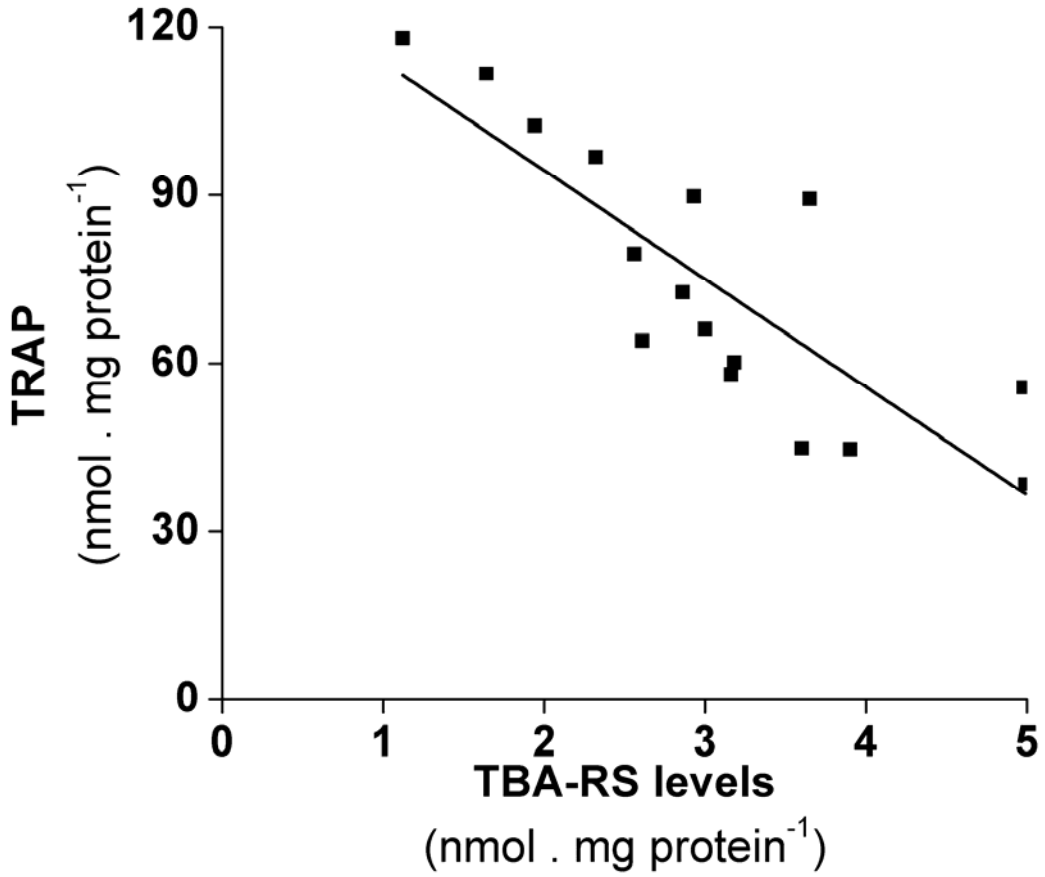
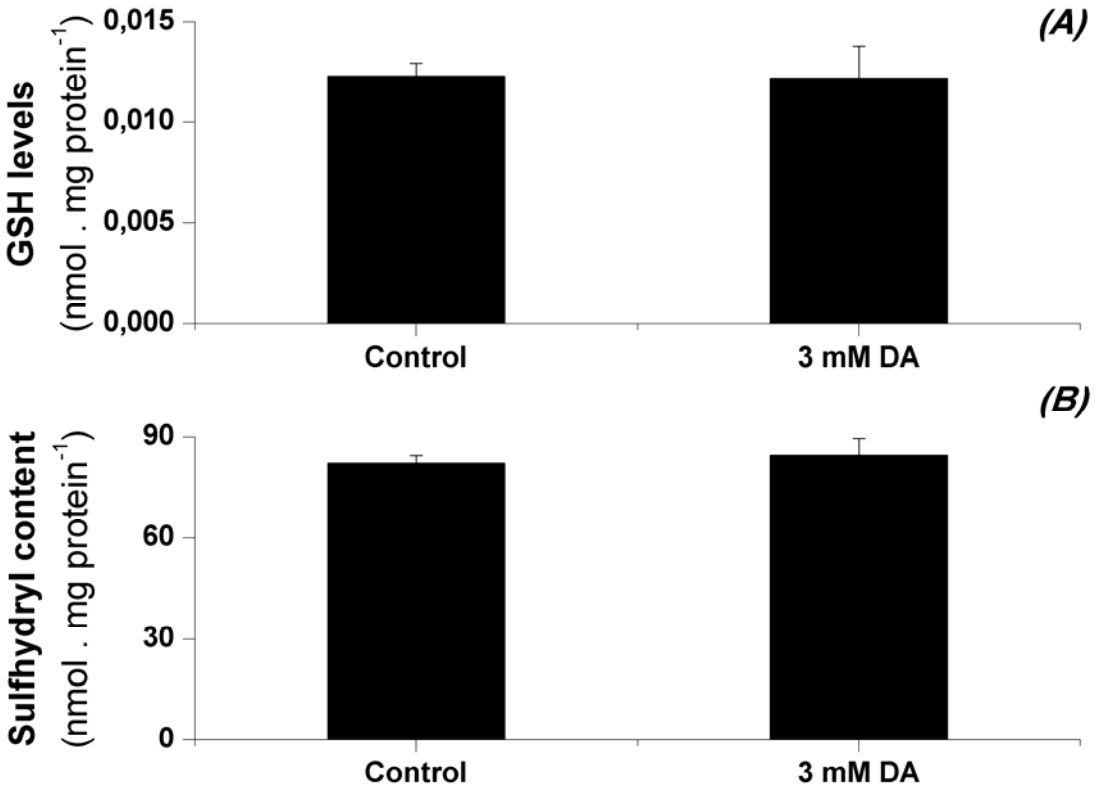

TRAP
(nmol . mg protein-1)
120
90
60
30
0
0
1
2
3
4
5
TBA-RS levels
(nmol . mg protein-1)

Figure 7.


(A)
GSH levels
(nmol . mg protein-1)
0,015
0,010
0,005
0,000
Control
3 mM DA
(B)
Sulfhydryl content
(nmol . mg protein-1)
90
60
30
0
Control
3 mM DA

PARTE III

Discussão e Conclusões

III.1. DISCUSSÃO

A deficiência da desidrogenase de acil-CoA de cadeia média (MCAD) é um dos erros inatos do metabolismo mais frequentes, tendo sua prevalência estimada em 1:6.500 a 1:17.000 recém-nascidos vivos (Rinaldo et al., 2002). As manifestações clínicas dos portadores dessa doença acometem principalmente o sistema nervoso central, podendo levar um número significativo de casos à morte súbita. No entanto, ainda pouco se sabe sobre os mecanismos fisiopatológicos do dano cerebral que acomete os pacientes portadores da deficiência de MCAD. Apesar de alguns autores apontarem a hipoglicemia e a síntese diminuída de corpos cetônicos como as principais causas do dano neurológico, a hipótese de que efeitos tóxicos causados pelos ácidos graxos acumulados na deficiência de MCAD estejam envolvidos vem sendo crescentemente considerada (Gregersen et al., 2008). Deve-se ainda enfatizar que várias manifestações clínicas desta doença ocorrem sem a presença de hipoglicemia, fortalecendo a hipótese de que os compostos acumulados possam atuar como agentes neurotóxicos.

Neste sentido, alguns estudos demonstraram efeitos tóxicos dos ácidos octanóico (AO), decanóico (AD) e *cis*-4-decenóico (AcD), os principais ácidos graxos acumulados na deficiência de MCAD (Roe e Ding, 2001). Foi verificado que a administração *in vivo* de AO e AD altera o transporte de ácidos orgânicos no plexo coróide, levando a um prejuízo na depuração deste ácido graxo e de outros compostos (Kim et al., 1983). Acredita-se que este efeito contribua para o acúmulo de ácidos graxos de cadeia média no cérebro e no liquido céfalo-raquidiano de pacientes afetados pela deficiência de MCAD. Também se observou que o AO

inibe o controle do volume de astrócitos e a atividade da enzima Na^+,K^+-ATPase em cultura de células gliais (Olson et al., 1989), o que poderia estar relacionado ao edema cerebral observado na doença. Demonstrou-se também que o AO causa um aumento no consumo de O_2 e na produção de CO_2, sem causar um correspondente aumento na produção de ATP em fígado de ratos, indicando que esse metabólito poderia provocar uma disfunção mitocondrial (Berry et al., 1983; Scholz et al., 1984).

Mais recentemente, foi demonstrado que os AO, AD e AcD inibem *in vitro* importantes parâmetros do metabolismo energético em cérebro de ratos jovens, tais como as atividades de complexos da cadeia transportadora de elétrons e das enzimas creatina quinase e Na^+,K^+-ATPase, bem como a produção de $^{14}CO_2$ a partir de [U-^{14}C]glicose, [1-^{14}C]acetato e [U-^{14}C] citrato (de Assis et al., 2003, 2006; Reis de Assis et al., 2004), corroborando uma alteração na homeostasia mitocondrial do cérebro na presença destes ácidos graxos.

Considerando que a mitocôndria desempenha papel central na geração de energia para a homeostasia dos processos celulares, através da manutenção dos níveis de ATP, que participa ativamente da homeostase de cálcio (Nicholls e Akerman, 1982) e que está envolvida em diversos processos que levam à morte celular, incluindo liberação de citocromo *c* (Liu et al., 1996), os capítulos I e II deste trabalho tiveram por objetivo avaliar o efeito dos ácidos AO, AD e AcD sobre alguns parâmetros de função mitocondrial.

Inicialmente, observamos que o AcD provocou um decréscimo no potencial de membrana mitocondrial ($\Delta\Psi m$) em mitocôndrias em estado 4 da respiração

(estado não-fosforilante), utilizando-se tanto uma combinação de glutamato e malato quanto succinato como substratos. Além disso, a adição de CCCP, um clássico desacoplador da fosforilação oxidativa, às preparações mitocondriais previamente tratadas com 1 mM de AcD não alterou o efeito apresentado por este ácido graxo, sugerindo uma ação desacopladora do AcD. Também observamos que o AcD diminuiu a produção de H_2O_2 quando succinato foi utilizado como substrato. Deve-se enfatizar que o H_2O_2 pode ser formado como um produto alternativo da cadeia respiratória, podendo ser liberado pela mitocôndria utilizando succinato como substrato de duas formas: pelo fluxo direto de elétrons (transferência de elétrons em direção ao complexo IV para reduzir O_2 a H_2O) ou pelo fluxo reverso de elétrons (elétrons provenientes do complexo II reduzem NAD^+ a NADH no complexo I passando pela CoQ, processo que ocorre em presença de alto potencial de membrana) (Nicholls e Ferguson, 2002). Na tentativa de elucidar o mecanismo pelo qual o AcD diminuiu a liberação de H_2O_2, adicionamos rotenona, em uma concentração que inibe totalmente o fluxo reverso de elétrons, em mitocôndrias respirando com succinato. Observamos que a rotenona não alterou o decréscimo de liberação de H_2O_2 causado pelo AcD, sugerindo que este ácido graxo inibe o fluxo reverso de elétrons. Entretanto, considerando que a rotenona *per se* ocasionou uma diminuição da liberação de H_2O_2, porém menor do que a causada pelo ácido *per se*, é possível que haja outros mecanismos além da inibição do fluxo reverso de elétrons pelos quais o AcD exerce seus efeitos na liberação de H_2O_2. Outras explicações poderiam ser um efeito desacoplador do ácido, inibição da transferência de elétrons e perda de NAD(P)H da matriz mitocondrial.

Nosso próximo passo foi avaliar o efeito do AcD sobre parâmetros respiratórios mitocondriais. Observamos que o ácido aumenta significativamente o estado 4 da respiração mitocondrial tanto com succinato quanto com glutamato/malato como substratos, reforçando a idéia de que este ácido graxo atue como um desacoplador da fosforilação oxidativa. Entretanto, o consumo de oxigênio no estado 4 com succinato foi menor na presença da concentração de 1 mM de AcD em comparação com a concentração de 0,5 mM, provavelmente devido a uma inibição do complexo II ocasionada pelo AcD, como demonstrado por Reis de Assis et al. (2004). Também observamos uma inibição na respiração mitocondrial estimulada por ADP (estado 3) apenas quando o succinato foi utilizado como substrato, confirmando, assim, a relevância da inibição da atividade do complexo II por este ácido. O AcD também diminuiu significativamente os valores do índice de controle respiratório (RCR) e a razão ADP/O de mitocôndrias, utilizando tanto glutamato/malato quanto succinato como substratos, indicando um prejuízo na eficiência da fosforilação oxidativa.

Muitos trabalhos demonstraram que o translocador de nucleotídeos adenina (ANT) está envolvido no efeito desacoplador de alguns ácidos graxos, e que neste caso esse efeito poderia ser prevenido pela co-incubação com atractilosídeo (ATC) (Brustovetskyl et al., 1990; Skulachev, 1998; Samartsev et al., 2000). Assim, na tentativa de esclarecer o mecanismo pelo qual o AcD exerce seu efeito desacoplador, testamos o efeito da co-incubação de AcD e ATC sobre o consumo de oxigênio. Observamos que o efeito desacoplador do AcD não foi alterado pela presença de ATC, indicando que este ácido graxo provavelmente atue por um mecanismo distinto de outros ácidos graxos, não interferindo no ANT. Uma

possível explicação para estes achados seria a de que o AcD afeta a bicamada lipídica da membrana mitocondrial, levando a uma alteração na fluidez, permeabilidade a íons e interação de fosfolipídeos (Kimmelberg e Pahadjopoulos, 1974; Lee, 1976; Abeywardena, Allen e Charnock, 1983).

Demonstramos também que o AcD reduziu significativamente o conteúdo de equivalentes reduzidos de NAD(P)H na matriz mitocondrial e que a posterior adição de rotenona ao meio de incubação aumentou esse conteúdo, embora não tenha atingido novamente os valores dos controles. Considerando que a rotenona reverte o efeito de desacopladores sobre o conteúdo de NAD(P)H, tais resultados poderiam ser devidos à perda de equivalentes reduzidos da matriz mitocondrial para o meio de incubação por um aumento na permeabilidade da membrana mitocondrial, provavelmente ocasionada por uma alteração na fluidez de membrana, como se observa com vários ácidos graxos (Kimmelberg e Pahadjopoulos, 1974; Lee, 1976; Abeywardena, Allen e Charnock, 1983). Outros experimentos do presente trabalho demonstraram que o AcD provocou um inchamento mitocondrial, o qual não foi impedido pela presença de ciclosporina A (CsA), um inibidor da abertura do poro de transição mitocondrial, excluindo um efeito do AcD sobre esta permeabilidade transitória. Esses resultados, associados à perda de NAD(P)H, sugerem que o AcD atua como desacoplador da fosforilação oxidativa através de um mecanismo relacionado a uma permeabilização não-seletiva da membrana mitocondrial interna.

Já no que diz respeito aos ácidos AO e o AD que também se acumulam na deficiência de MCAD, os mesmos apresentaram um efeito desacoplador da fosforilação oxidativa, diminuindo o $\Delta\Psi m$ de mitocôndrias respirando com

glutamato/malato ou com succinato. A posterior adição de CCCP não aumentou o efeito da concentração mais alta (3 mM) de AD, mostrando que nesta concentração este ácido graxo exerce um efeito desacoplador comparável ao de um desacoplador clássico. Já o AO apresentou um efeito semelhante, porém de menor intensidade, sendo que o CCCP foi capaz de reduzir ainda mais o potencial de membrana mitocondrial.

Nosso próximo passo foi investigar o efeito do AO e AD sobre os parâmetros respiratórios medidos por oximetria. Observamos que ambos ácidos graxos aumentaram o estado 4 tanto com glutamato/malato quanto com succinato como substratos, reforçando a hipótese de que eles atuem como desacopladores da fosforilação oxidativa. Da mesma forma que o AcD, o consumo de oxigênio no estado 4 com succinato foi menor na presença da concentração de 3 mM de AD em comparação com a concentração de 1 mM com ambos os substratos. Entretanto, diferentemente do AcD, estudos prévios demonstraram que o AD não altera a atividade do complexo II da cadeia transportadora de elétrons (Reis de Assis et al., 2004). Tal resultado sugere um efeito diferencial do AD em relação aos do AO e do AcD. Observamos também que tanto o AD inibiu o estado 3 da respiração mitocondrial com quaisquer dos substratos testados, enquanto que o AO inibiu apenas quando succinato foi utilizado como substrato. Estes ácidos graxos também provocaram uma diminuição do RCR, reforçando a idéia de uma ação desacopladora destes ácidos, mas apenas o AD diminuiu a razão ADP/O, indicando um prejuízo na eficiência da fosforilação oxidativa. Com base nestes dados, podemos sugerir que estes ácidos graxos exerçam seus efeitos através de mais de um mecanismo. Possivelmente ao efeito desacoplador da fosforilação

oxidativa soma-se uma interferência em algum dos processos mitocondriais de oxidação, tais como efeito sobre o ANT, inibição de transportadores de substratos ou de atividade dos complexos da cadeia transportadora de elétrons.

Observamos também que o efeito causado pelo AO não foi alterado pela presença de ATC. Todavia, o efeito do AD aumentando o estado 4 foi atenuado pelo ATC, sugerindo que este ácido graxo possa atuar através de um mecanismo envolvendo o ANT.

Por outro lado, um comprometimento no transporte através das membranas mitocondriais ou uma inibição da atividade de complexos da cadeia transportadora de elétrons também poderiam explicar os efeitos causados pelo AO e AD sobre o estado 3 da respiração, bem como sobre os valores do RCR e da razão ADP/O.

O próximo passo foi avaliar o efeito do AO e do AD sobre o sistema oxidante (transporte de substratos através de membranas, atividades de desidrogenases e a função da cadeia transportadora de elétrons) através da medida do consumo de oxigênio em mitocôndrias desacopladas (estado U), descartando a interferência no sistema fosforilante (ATPsintase, ANT, transportadores de fosfato e vazamento de prótons pela membrana). Observamos que tanto o AO quanto o AD diminuíram o consumo de oxigênio nessas condições, indicando que outros mecanismos além de efeito desacoplador estão envolvidos nos efeitos induzidos por estes ácidos graxos de cadeia média.

Avaliamos então o efeito do AO e do AD sobre atividades dos complexos da cadeia transportadora de elétrons e observamos que ambos os ácidos graxos inibiram as atividades dos complexos I-III e IV. Além disso, apenas o AD inibiu a atividade do complexo II-III. Portanto os efeitos do AO e do AD interferindo na taxa

de respiração nos estados estimulado e não-estimulado por ADP podem ser parcialmente explicados pela inibição da transferência de elétrons através da cadeia respiratória. Porém não podemos descartar um efeito do AO e AD sobre o transporte de substratos (trocadores de glutamato/aspartato e transportador de succinato), atividades de desidrogenases na matriz mitocondrial ou liberação de citocromo *c*, o que poderia causar os mesmos achados.

Observamos ainda que o AO e o AD diminuíram significativamente o conteúdo de equivalentes reduzidos de NAD(P)H na matriz mitocondrial e que a posterior adição de rotenona ao meio de incubação reverteu totalmente a diminuição causada pelo AO, mas reverteu apenas parcialmente o efeito do AD. Da mesma forma que o AcD, tais resultados encontrados com o AD sugerem que estes equivalentes reduzidos possam estar sendo perdidos da matriz mitocondrial para o meio de incubação. Também observamos que apenas o AD ocasionou um inchamento mitocondrial. Esses resultados, associados à perda de NAD(P)H, sugerem que este ácido graxo, similarmente ao que ocorre com o AcD, atue como desacoplador da fosforilação oxidativa através de um mecanismo relacionado a uma permeabilização não-seletiva da membrana mitocondrial interna.

Através dos resultados demonstrados nos capítulos I e II deste trabalho, podemos concluir que os principais ácidos graxos de cadeia média acumulados na deficiência de MCAD, os ácidos octanóico, decanóico e *cis*-4-decenóico, ocasionam um distúrbio na homeostasia mitocondrial, provavelmente levando a um déficit energético. Devemos enfatizar, no entanto, que estes compostos, apesar de provocarem desacoplamento e inibição metabólica, parecem atuar via mecanismos múltiplos e distintos.

Também objetivamos no presente trabalho (capítulos III e IV) investigar os efeitos dos AO, AD e AcD sobre alguns parâmetros de estresse oxidativo em córtex cerebral de ratos jovens. Enfatize-se que alterações mitocondriais, especialmente inibições de complexos da cadeia respiratória, podem levar a um aumento na geração de espécies reativas (Halliwell e Gutteridge, 2007). Observamos que o AcD, em concentrações de 0,5 e 1 mM, ocasionou um aumentou na peroxidação lipídica, evidenciado por um aumento na quimioluminescência espontânea e nos níveis de substâncias reativas ao ácido tiobarbitúrico (TBA-RS). O aumento nos níveis de TBA-RS foi evidenciado já com 30 minutos de pré-incubação, na concentração de 1 mM de AcD. No intuito de verificar o envolvimento de espécies reativas neste aumento da lipoperoxidação, avaliamos o efeito de alguns sequestradores de espécies reativas e antioxidantes sobre o aumento dos níveis de TBA-RS causado pelo AcD. Observamos que a taurina, a creatina e o gangliosídio GM1, bem como altas doses de glutationa reduzida (GSH), trolox (TRO) e uma combinação das enzimas antioxidantes catalase (CAT) e superóxido dismutase (SOD) foram capazes de prevenir o efeito causado pelo AcD, sugerindo o envolvimento de espécies reativas de oxigênio neste efeito, especialmente radicais peroxil e alcoxil, H_2O_2 e/ou ânion superóxido, principais espécies reativas atacadas por TRO, CAT e SOD, respectivamente (Halliwell e Gutteridge, 2007). Entretanto, quando avaliamos os níveis de TBA-RS em preparações mitocondriais, o AcD não foi capaz de alterar este parâmetro, sugerindo que não há envolvimento mitocondrial nos mecanismos pelos quais este ácido graxo ocasiona um aumento da lipoperoxidação, ou então que há necessidade de componentes citosólicos para que tal ação ocorra. Também

observamos que o AcD aumentou a formação de carbonilas e a oxidação de grupamentos tióis, sugerindo que este composto gera um dano oxidativo a proteínas. Considerando que o conteúdo aumentado de carbonilas é um indicador de dano oxidativo a proteínas, e que a maior parte dos grupamentos sulfidrilas é ligada a proteínas (Reznick and Packer, 1994; Levine, 2002), pode-se afirmar que o AcD provoca dano oxidativo proteico. Verificamos ainda que este ácido graxo ocasionou uma maior oxidação da 2',7'-di-hidrodiclorofluoresceína, um indicativo de aumento na geração de espécies reativas. Por outro lado, o AcD ocasionou uma diminuição nas defesas antioxidantes não-enzimáticas, refletida pela redução no potencial antioxidante total do tecido (TRAP), na reatividade antioxidante do tecido (TAR) e nos níveis de GSH, a principal defesa antioxidante do cérebro, sem alterar a atividade das enzimas antioxidantes catalase, superóxido dismutase e glutationa peroxidase. A diminuição dos níveis de GSH provocada pelo AcD foi prevenida pela adição de melatonina (MEL) e TRO, reforçando a idéia de um envolvimento de espécies reativas nos efeitos do AcD, principalmente de radicais peroxil, alcoxil, hidroxil, bem como das espécies reativas de nitrogênio óxido nítrico e ânion peroxinitrito, principais espécies reativas sequestradas por MEL e TRO (Halliwell e Gutteridge, 2007; Maharaj et al., 2007). Na tentativa de determinar se houve envolvimento de ânions superóxido no dano oxidativo provocado por este ácido graxo, avaliamos o efeito do AcD sobre a produção desta espécie reativa em partículas submitocondriais, e não evidenciamos nenhum efeito do composto sobre a produção de ânion superóxido.

Observamos ainda uma correlação positiva entre os valores de TBA-RS e de TRAP, reforçando os resultados de que a diminuição das defesas antioxidantes

não-enzimáticas cerebrais foram devidas a um consumo dos antioxidantes secundário a um aumento na geração de espécies reativas provocado pelo AcD.

Da mesma forma, avaliamos os efeitos do AO e do AD sobre alguns parâmetros de estresse oxidativo em córtex cerebral de ratos, o que foi objetivo do capítulo IV deste trabalho. Observamos que tanto o AO quanto o AD induziram um aumento na lipoperoxidação, evidenciado por um aumento nos níveis de TBA-RS e na medida da quimioluminescência espontânea, refletindo efeitos mais pronunciados do AD. Este ácido graxo foi também capaz de causar este aumento nos níveis de TBA-RS a partir de 30 minutos de pré-incubação, enquanto o AO foi capaz de exercer seu efeito apenas após 60 minutos de pré-incubação. Considerando que o AD exerceu um efeito mais pronunciado sobre os níveis de TBA-RS e com o objetivo de verificar o envolvimento de espécies reativas neste aumento da lipoperoxidação causado pelo AD, avaliamos o efeito de alguns sequestradores de espécies reativas e antioxidantes sobre o aumento dos níveis de TBA-RS causado pelo AD. Nossos resultados demonstraram que TRO e MEL foram capazes de prevenir totalmente o aumento nos níveis de TBA-RS ocasionado pelo AD, enquanto que GSH foi capaz de prevenir apenas parcialmente este efeito. Estes resultados reforçam a idéia de que esse efeito é mediado por espécies reativas. Considerando que o TRO é efetivo contra radicais peroxil e alcoxil (Halliwell and Gutteridge, 2007) e que a MEL atua principalmente contra os radicais hidroxil e peroxil e as espécies reativas de nitrogênio óxido nítrico e ânion peroxinitrito (Maharaj et al., 2007), pode-se especular que essas espécies reativas estejam envolvidas na lipoperoxidação exercida pelo AD. Também observamos um aumento no conteúdo de carbonilas e na oxidação de

grupamentos tióis (parâmetros de dano oxidativo protéico) causadas apenas pelo AD, sugerindo que este ácido graxo atua de forma distinta do AO. Observamos também que os AO e AD não alteraram o conteúdo de GSH em uma solução comercial de GSH sem a presença de homogeneizados cerebrais, sugerindo que estes compostos não atuam diretamente como agentes pró-oxidantes *per se*.

Em relação às defesas antioxidantes não-enzimáticas, observamos que ambos os ácidos graxos testados diminuíram o TRAP, mas apenas o AD diminuiu significativamente os níveis de GSH no tecido. Tal resultado sugere que o AO atua em outras defesas antioxidantes não-enzimáticas do que sobre o GSH. A MEL e o TRO também foram capazes de prevenir o efeito do AD sobre os níveis de GSH, corroborando a idéia de que as principais espécies envolvidas neste efeito sejam os radicais peroxil, alcoxil e hidroxil e as espécies reativas de nitrogênio óxido nítrico e ânion peroxinitrito. Entretanto, descartamos o envolvimento de espécies reativas de nitrogênio, visto que este ácido graxo não alterou os níveis de nitratos e nitritos.

Visto que níveis adequados de antioxidantes são essenciais para a proteção da célula contra dano oxidativo, e que um desequilíbrio na homeostase entre pró-oxidantes e antioxidantes induz estresse oxidativo, é possível que a diminuição significativa dos níveis de GSH ocasionada pelo AD esteja relacionada ao aumento da lipoperoxidação ocasionado pelo mesmo composto. Por outro lado, não podemos descartar a possibilidade de o decréscimo nos níveis de GSH se deva a um aumento na geração de espécies reativas causado pelo AD. Neste contexto, obtivemos uma forte correlação inversa entre o aumento dos níveis de TBA-RS e a diminuição do TRAP ocasionados pelo AD, indicando que estes

efeitos estão inter-relacionados. Portanto, é possível que o decréscimo nas defesas antioxidantes não-enzimáticas reflita um rápido consumo nos antioxidantes endógenos devido a um aumento na produção de espécies reativas.

Na tentativa de elucidar os mecanismos pelos quais o AD exerce seus efeitos pró-oxidantes, avaliamos o efeito do AD sobre os níveis de GSH e o conteúdo de grupamentos tióis em preparações citosólicas, isto é, na ausência de mitocôndrias. Observamos que, nestas condições, o AD não foi capaz de exercer seus efeitos. Tal resultado, aliado ao fato de que a mitocôndria é a principal fonte de espécies reativas na célula (Cadenas e Davies, 2000; Halliwell e Gutteridge, 2007), sugere um papel para a mitocôndria nos mecanismos pelos quais o AD exerce seus efeitos tóxicos. Alternativamente, poder-se-ia supor que o maquinário celular deve estar completo (homogeneizado total) para que os efeitos pró-oxidantes do AD sejam observados.

Considerando que o estresse oxidativo é resultado de um desequilíbrio entre as defesas antioxidantes totais do tecido e a geração de espécies reativas, nossos resultados em seu conjunto demonstram que AcD, AO e AD induzem *in vitro* estresse oxidativo em córtex cerebral de ratos, um condição deletéria para a célula. Faz-se importante enfatizar que o cérebro possui menos defesas antioxidantes (Halliwell e Gutteridge, 1996), o que o torna mais vulnerável ao ataque de espécies reativas.

Tomados em seu conjunto, as presentes observações indicam claramente que o AcD foi o metabólito que provocou as alterações mais pronunciadas, tanto na homeostasia energética mitocondrial, quanto no dano oxidativo, visto que o mesmo exerceu efeitos significativos em concentrações menores relativamente ao

AD e ao AO. Também observamos que o AD teve um efeito mais pronunciado do que o do AO nas mesmas concentrações sobre os parâmetros analisados no presente estudo. Tais resultados podem dever-se ao fato de que ácidos graxos insaturados provocam alterações mais importantes na permeabilidade de membranas celulares ao interferir com as interações entre os fosfolipídeos de membrana (Kimmelberg e Pahadjopoulos, 1974; Lee, 1976; Abeywardena, Allen e Charnock, 1983). Outro aspecto que pode ser relevante é o tamanho da cadeia carbonada, visto que resultados prévios demonstrando que quanto maior a cadeia de carbono desses ácidos maior é o seu efeito sobre a membrana (Kimmelberg e Pahadjopoulos, 1974; Lee, 1976; Abeywardena, Allen e Charnock, 1983).

Apesar de não podermos precisar a relevância fisiopatológica dos presentes achados, deve-se enfatizar que os efeitos tóxicos aqui apresentados ocorreram em concentrações similares àquelas encontradas em tecidos de pacientes afetados pela deficiência de MCAD durante as crises metabólicas em que ocorre um grande aumento nas concentrações teciduais dos ácidos graxos de cadeia média acumulados nesta doença.

Tem sido presumido que, em várias acidemias orgânicas, as concentrações dos ácidos orgânicos acumulados nas células neurais são maiores do que as encontradas no plasma e no líquido céfalo-raquidiano nos pacientes afetados por estas doenças (Hoffmann et al., 1993). Este parece ser o caso na deficiência de MCAD, visto que a administração *in vivo* de AO em ratos altera o transporte de ácidos orgânicos pelo plexo coróide, levando a um prejuízo na depuração transcoroidal de AO e compostos similares (Kim et al., 1983). Foi, então, sugerido que este mecanismo contribuiria para o acúmulo de ácidos graxos de cadeia

media no cérebro e no líquido céfalo-raquidiano em pacientes acometidos pela síndrome de Reye, uma desordem em que também ocorre acúmulo da AO. Por outro lado, deve-se considerar que as enzimas envolvidas na oxidação de ácidos graxos, incluindo a MCAD, são expressas no cérebro (Tyni et al., 2004), o que reforça a idéia de que ácidos graxos de cadeia média possam se acumular no cérebro dos pacientes afetados por esta desordem.

Os presentes resultados indicam que os principais metabólitos acumulados na deficiência de MCAD exercem efeitos neurotóxicos importantes e que os sintomas característicos dessa doença não podem ser exclusivamente devidos à hipoglicemia e diminuição da síntese de corpos cetônicos. Dessa forma, é possível que uma disfunção mitocondrial e o estresse oxidativo, possivelmente aliados a outros mecanismos, atuem sinergicamente, levando ao dano neurológico apresentado pelos pacientes afetados pela deficiência de MCAD. Acreditamos, portanto, que os achados deste trabalho possam auxiliar na elucidação dos mecanismos fisiopatogênicos da neurodegeneração característica dos pacientes com esta doença, servindo como base para o desenvolvimento de estratégias terapêuticas relevantes no tratamento e na melhora da qualidade de vida dos portadores da deficiência de MCAD.

III.2. CONCLUSÕES

III.2.1. Efeitos dos ácidos AcD, AO e AD sobre a homeostase mitocondrial

- Os ácidos *cis*-4-decenóico (AcD), octanóico (AO) e decanóico (AD) diminuíram o potencial de membrana mitocondrial em preparações mitocondriais de cérebro de ratos de 30 dias de vida quando glutamato/malato ou succinato foram utilizados como substratos, comportando-se como desacopladores da fosforilação oxidativa.

- O AcD diminuiu a produção de H_2O_2 a partir de succinato em preparações mitocondriais de cérebro dos ratos.

- Os ácidos AO, AD e AcD aumentaram o estado 4 e diminuíram o índice de controle respiratório (RCR), tanto quando glutamato/malato como quando succinato foram utilizados como substratos nas preparações mitocondriais de cérebro de ratos.

- O AcD e o AD diminuíram o estado 3 da respiração mitocondrial tanto com glutamato/malato quanto com succinato como substratos em preparações mitocondriais de cérebro dos ratos. Entretanto, o AO diminuiu o estado 3 da respiração mitocondrial apenas quando succinato foi utilizado como substrato.

- O AcD e o AD diminuíram a razão ADP/O em preparações mitocondriais de cérebro de ratos.

- O AO e o AD inibiram o consumo de oxigênio em mitocôndrias desacopladas de cérebro de ratos.

- O translocador de nucleotídeos de adenina está envolvido no efeito desacoplador do AD, visto que a presença de atractilosídeo reverteu parcialmente o efeito desacoplador desse ácido.
- O AO e o AD inibiram a atividade dos complexos I-III e IV da cadeia transportadora de elétrons em preparações mitocondriais de cérebro de ratos, enquanto apenas o ácido decanóico inibiu o complexo II-III.
- Os ácidos AO, AD e AcD diminuíram o conteúdo dos equivalentes reduzidos de NADH e NADPH em preparações mitocondriais de cérebro de ratos.
- Os ácidos decanóico (AD) e *cis*-4-decenóico (AcD) causaram inchamento mitocondrial em preparações mitocondriais de cérebro de ratos.

III.2.2. Efeitos dos AcD, AO e AD sobre parâmetros de estresse oxidativo

- Os ácidos *cis*-4-decenóico (AcD), octanóico (AO) e decanóico (AD), induziram dano oxidativo lipídico, evidenciado pelo aumento dos níveis de substâncias reativas ao ácido tiobarbitúrico e da quimioluminescência espontânea em homogeneizado de córtex cerebral de ratos de 30 dias de vida.
- O aumento nos níveis de substâncias reativas ao ácido tiobarbitúrico causado pelo AcD foi prevenido co-incubação com GM1, taurina, creatina, glutationa reduzida, trolox e uma combinação das enzimas antioxidantes catalase e superóxido dismutase.
- O aumento nos níveis de substâncias reativas ao ácido tiobarbitúrico causado pelo AD foi prevenido pela co-incubação com trolox e melatonina, e parcialmente prevenido por glutationa reduzida.

- Os ácidos AcD, AO e AD induziram dano oxidativo proteico, evidenciado pelo aumento no conteúdo de carbonilas e diminuição do conteúdo de grupamentos tióis em homogeneizado de córtex cerebral de ratos.
- Os ácidos AcD, AO e AD diminuíram as defesas antioxidantes não-enzimáticas, evidenciado pela diminuição no potencial antioxidante total do tecido (TRAP), enquanto o AcD e o AD também reduziram as concentrações de glutationa reduzida em homogeneizado de córtex cerebral de ratos.
- O AcD diminuiu a reatividade antioxidante do tecido (TAR) em homogeneizado de córtex cerebral de ratos.
- O AcD não alterou os níveis de substâncias reativas ao ácido tiobarbitúrico em preparações mitocondriais de cérebro de ratos, sugerindo um mecanismo extramitocondrial para os efeitos causados por este ácido graxo.
- O AcD não alterou o conteúdo de ânion superóxido em partículas submitocondriais de córtex cerebral de ratos, indicando que esta espécie reativa não está envolvida nos efeitos mediados por este ácido graxo.
- O AcD aumentou a oxidação da 2',7'-di-hidrodiclorofluoresceína (DCFH) em homogeneizado de córtex cerebral de ratos, sugerindo o envolvimento de espécies reativas nos efeitos ocasionados por este ácido graxo.
- O AcD não alterou a atividade das enzimas antioxidantes catalase, superóxido dismutase e glutationa peroxidase em homogeneizado de córtex cerebral de ratos.
- O AO e o AD não alteraram o conteúdo de nitratos e nitritos em homogeneizado de córtex cerebral de ratos, sugerindo que espécies reativas de

nitrogênio não estão envolvidas nos efeitos pró-oxidantes desencadeados por estes ácidos graxos.

- O AD não alterou o conteúdo de grupamentos tióis e os níveis de glutationa reduzida em preparações citosólicas de córtex cerebral de ratos, sugerindo um envolvimento da mitocôndria nos efeitos causados por este ácido.

Esses resultados no seu conjunto indicam que os ácidos AcD, AD e AO causam um prejuízo à homeostase mitocondrial e induzem estresse oxidativo, e poderiam participar dos mecanismos fisiopatológicos que levam ao dano neurológico característico da deficiência de MCAD.

II.3. PERSPECTIVAS

- Avaliar o efeito *in vitro* dos ácidos octanóico (AO) e decanóico (AD) sobre o transporte de substratos para dentro da mitocôndria em preparações mitocondriais de cérebro de ratos jovens;
- Avaliar o efeito *in vitro* dos ácidos octanóico (AO), decanóico (AD) e *cis*-4-decenóico (AcD) sobre a liberação de citocromo *c* em preparações mitocondriais de cérebro de ratos jovens;
- Avaliar o efeito *in vitro* dos ácidos octanóico (AO), decanóico (AD) e *cis*-4-decenóico (AcD) sobre a fluidez de membrana mitocondrial em preparações mitocondriais de cérebro de ratos jovens;
- Avaliar um possível efeito sinérgico dos ácidos octanóico (AO), decanóico (AD) e *cis*-4-decenóico (AcD) *in vitro* sobre parâmetros de estresse oxidativo e função mitocondrial em cérebro de ratos jovens;
- Avaliar o efeito da administração aguda (intracerebral) e crônica (intraperitoneal) dos ácidos octanóico (AO) e decanóico (AD) sobre parâmetros de metabolismo energético, estresse oxidativo e função mitocondrial em diferentes estruturas e tecidos de ratos jovens;
- Avaliar o efeito *in vitro* de outros metabólitos acumulados na deficiência de MCAD, tais como conjugados de glicina e carnitina, sobre parâmetros de metabolismo energético, estresse oxidativo e função mitocondrial em cérebro de ratos jovens.

III.4. REFERÊNCIAS BIBLIOGRÁFICAS

Abeles RH, Frey PA, Jenks WP. Biochemistry. London: Jones and Bartlett, 603-632, 1992.

Abeywardena MY, Allen TM, Charnock JS. Lipid-protein interactions of reconstituted membrane-associated adenosine triphosphatases. Biochim Biophys Acta 729:62–74, 1983.

Alexi T, Borlongan CV, Faull RL, Williams CE, Clark RG, Gluckman PD, Hughes PE. Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. Prog Neurobiol, 409-70, 2000.

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

Barschak AG, Marchesan C, Sitta A, Deon M, Giugliani R, Wajner M, Vargas CR. Maple syrup urine disease in treated patients: biochemical and oxidative stress profiles. Clin Biochem 41:317-24, 2008a.

Barschak AG, Sitta A, Deon M, Barden AT, Dutra-Filho CS, Wajner M, Vargas CR. Oxidative stress in plasma from maple syrup urine disease patients during treatment. Metab Brain Dis 23:71-80, 2008b.

Bauché F, Sabourault D, Giudicelli Y, Nordmann J, Nordmann R. 2-mercaptoacetate administration depresses the beta-oxidation pathway through an inhibition of long-chain acyl-CoA dehydrogenase activity. Biochem J 196:803-9, 1981.

Beal MF. Mitochondria and neurodegeneration. Novartis Found Symp 287:183-92, 2007.

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

Ben-Shachar D, Karry R. Neuroanatomical pattern of mitochondrial complex I pathology varies between schizophrenia, bipolar disorder and major depression. PLoS ONE 3:e3676, 2008.

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

Berry MN, Clark DG, Grivell AR, Wallace PG. The calorigenic nature of hepatic ketogenesis: an explanation for the stimulation of respiration induced by fatty acid substrates. Eur J Biochem 131:205-14, 1983.

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

Boveris A. Biochemistry of free radicals: from electrons to tissues. Medicina (B Aires) 58:350-6, 1998.

Brustovetskyl NN,Dedukhova VI, Egoroval MV, Mokhova EN, Skulachev VP. Inhibitors of the ATP/ADP antiporter suppress stimulation of mitochondrial respiration and H+ permeability by palmitate and anionic detergents. FEBS Lett 272:187-189, 1990.

Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med 29:222-30, 2000.

Chace DH, Hillman SL, Van Hove JL, Naylor EW. Rapid diagnosis of MCAD deficiency: quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood spots by tandem mass spectrometry. Clin Chem 43:2106-13, 1997.

Costa CG, Guérand WS, Struys EA, Holwerda U, ten Brink HJ, Tavares de Almeida I, Duran M, Jakobs C. Quantitative analysis of urinary acylglycines for the diagnosis of beta-oxidation defects using GC-NCI-MS. J Pharm Biomed Anal 21:1215-24, 2000.

de Assis DR, Maria RC, Ferreira GC, Schuck PF, Latini A, Dutra-Filho CS, Wannmacher CM, Wyse AT, Wajner M. Na^+,K^+-ATPase activity is markedly reduced by *cis*-4-decenoic acid in synaptic plasma membranes from cerebral cortex of rats. Exp Neurol 197:143-9, 2006.

de Assis DR, Ribeiro CA, Rosa RB, Schuck PF, Dalcin KB, Vargas CR, Wannmacher CM, Dutra-Filho CS, Wyse AT, Briones P, Wajner M.Evidence that antioxidants prevent the inhibition of Na^+,K^+-ATPase activity induced by octanoic acid in rat cerebral cortex in vitro. Neurochem Res 28:1255-63, 2003.

Del Prete E, Lutz TA, Althaus J, Scharrer E. Inhibitors of fatty acid oxidation (mercaptoacetate,R-3-amino-4-trimethylaminobutyric acid) stimulate feeding in mice. Physiol Behav 63:751-4, 1998.

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

Deon M, Garcia MP, Sitta A, Barschak AG, Coelho DM, Schimit GO, Pigatto M, Jardim LB, Wajner M, Giugliani R, Vargas CR. Hexacosanoic and docosanoic acids plasma levels in patients with cerebral childhood and asymptomatic X-linked adrenoleukodystrophy: Lorenzo's oil effect. Metab Brain Dis 23:43-9, 2008.

Deon M, Sitta A, Barschak AG, Coelho DM, Pigatto M, Schmitt GO, Jardim LB, Giugliani R, Wajner M, Vargas CR.Induction of lipid peroxidation and decrease of antioxidant defenses in symptomatic and asymptomatic patients with X-linked adrenoleukodystrophy. Int J Dev Neurosci 25:441-4, 2007.

Derks TG, Reijngoud DJ, Waterham HR, Gerver WJ, van den Berg MP, Sauer PJ, Smit GP. The natural history of medium-chain acyl CoA dehydrogenase deficiency in the Netherlands. J Pediatr 148:665-670, 2006.

Derks TG, van Dijk TH, Grefhorst A, Rake JP, Smit GP, Kuipers F, Reijngoud DJ. Inhibition of mitochondrial fatty acid oxidation in vivo only slightly suppresses gluconeogenesis but enhances clearance of glucose in mice. Hepatology 47:1032-42, 2008.

Di Donato S. Disorders related to mitochondrial membranes: pathology of the respiratory chain and neurodegeneration, J Inherit Metab Dis 23:247– 263, 2000.

Downing M, Rose P, Bennett MJ, Manning NJ, Pollitt RJ. Generalised dicarboxylic aciduria: a common finding in neonates. J Inherit Metab Dis 12:321-4, 1989.

Egidio RJ, Francis GL, Coates PM, Hale DE, Roesel A. Medium-chain acyl-CoA dehydrogenase deficiency, Am Fam Physician 39:221-6, 1989.

Feksa LR, Latini A, Rech VC, Feksa PB, Koch GD, Amaral MF, Leipnitz G, Dutra-Filho CS, Wajner M, Wannmacher CM. Tryptophan administration induces oxidative stress in brain cortex of rats. Metab Brain Dis 23:221-33, 2008.

Ferreira GC, Tonin A, Schuck PF, Viegas CM, Ceolato PC, Latini A, Perry ML, Wyse AT, Dutra-Filho CS, Wannmacher CM, Vargas CR, Wajner M. Evidence for a synergistic action of glutaric and 3-hydroxyglutaric acids disturbing rat brain energy metabolism. Int J Dev Neurosci 25:391-8, 2007.

Freund K, Mizzer J, Dick W, Thorpe C. Inactivation of general acyl-CoA dehydrogenase from pig kidney by 2-alkynoyl coenzyme A derivatives: initial aspects. Biochemistry 24:5996-6002, 1985.

Gardner A, Salmaso D, Nardo D, Micucci F, Nobili F, Sanchez-Crespo A, Jacobsson H, Larsson SA, Pagani M. Mitochondrial function is related to alterations at brain SPECT in depressed patients. CNS Spectr 13:805-814, 2008.

Gil JM, Rego AC. Mechanisms of neurodegeneration in Huntington's disease. Eur J Neurosci 28:2156, 2008.

Gregersen N, Andresen BS, Pedersen CB, Olsen RK, Corydon TJ, Bross P. Mitochondrial fatty acid oxidation defects-remaining challenges. J Inherit Metab Dis 31:643-657, 2008.

Grosse SD, Khoury MJ, Greene CL, Crider KS, Pollitt RJ. The epidemiology of medium-chain acyl-CoA dehydrogenase deficiency: An update. Genet Med 8:205-212, 2006.

Gu M, Owen AD, Toffa SE, Cooper JM, Dexter DT, Jenner P, Marsden CD, Schapira AH. Mitochondrial function, GSH and iron in neurodegeneration and Lewy body diseases. J Neurol Sci 158:24-9, 1998.

Halliwell B, Gutteridge JMC. (Ed.) Free Radicals in Biology and Medicine. Oxford University Press, Oxford, 614-677, 2007.

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

Halliwell B, Whiteman M. Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? Br J Pharmacol 142:231-55, 2004.

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

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

Han D, Williams E, Cadenas E. Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. Biochem J 353:411-6, 2001.

Hoffmann GF, Seppel CK, Holmes B, Mitchell L, Christen HJ, Hanefeld F, Rating D, Nyhan WL. Quantitative organic acid analysis in cerebrospinal fluid and plasma: reference values in a pediatric population.J Chromatogr 617:1-10, 1993.

Janetzky B, Hauck S, Youdim MB, Riederer P, Jellinger K, Pantucek F, Zöchling R, Boissl KW, Reichmann H. Unaltered aconitase activity, but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. Neurosci Lett 169:126-8, 1994.

Kessler A, Biasibetti M, da Silva Melo DA, Wajner M, Dutra-Filho CS, de Souza Wyse AT, Wannmacher CM. Antioxidant effect of cysteamine in brain cortex of young rats. Neurochem Res 33:737-44, 2008.

Kim CS, Dorgan DR, Roe CR. L-carnitine: therapeutic strategy for metabolic encephalopathy. Brain Res 310:149-53, 1984.

Kim CS, O'tuama LA, Mann JD, Roe CR. Effect of increasing carbon chain length on organic acid transport by the choroid plexus: a potential factor in Reye's syndrome. Brain Res 259:340–343, 1983.

Kim CS, Roe CR, Ambrose WW, Ll-Carnitine prevents mitochondrial damage induced by octanoic acid in the rat choroid plexus. Brain Res 536:335–338, 1990.

Kimmelberg H, Pahadjopoulos D. Effects of phospholipid acyl chain fluidity, phase transitions, and cholesterol on (Na+,K+)-stimulated adenosine triphosphatase. J Biol Chem 249:1071–1080, 1974.

Kølvraa S, Gregersen N, Christensen E, Hobolth N. *In vitro* fibroblast studies in a patient with C6-C10-dicarboxylic aciduria: evidence for a defect in general acyl-CoA dehydrogenase. Clin Chim Acta 126:53-67, 1982.

Kompare M, Rizzo WB. Mitochondrial fatty-acid oxidation disorders. Seminars in Pediatric Neurology 15:149-149, 2008.

Latini A, da Silva CG, Ferreira GC, Schuck PF, Scussiato K, Sarkis JJ, Dutra Filho CS, Wyse AT, Wannmacher CM, Wajner M. Mitochondrial energy metabolism is markedly impaired by D-2-hydroxyglutaric acid in rat tissues. Mol Genet Metab 86:188-99, 2005.

Latini A, Scussiato K, Leipnitz G, Gibson KM, Wajner M. Evidence for oxidative stress in tissues derived from succinate semialdehyde dehydrogenase-deficient mice. J Inherit Metab Dis 30:800-10, 2007.

Lee AG. Model for action of local anesthetics. Nature 262:545–548, 1976.

Lee PJ, Harrison EL, Jones MG, Jones S, Leonard JV, Chalmers RA. L-carnitine and exercise tolerance in medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency: a pilot study. J Inherit Metab Dis 28:141-52, 2005.

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

Levine RL. Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radic Biol Med 32:790–6, 2002.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86:147-57, 1996.

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

Maegawa GH, Poplawski NK, Andresen BS, Olpin SE, Nie G, Clarke JT, Teshima I. Interstitial deletion of 1p22.2p31.1 and medium-chain acyl-CoA dehydrogenase deficiency in a patient with global developmental delay. Am J Med Genet 146:1581-6, 2008.

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

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

Matsubara Y, Narisawa K, Tada K. Medium-chain acyl-CoA dehydrogenase deficiency: molecular aspects. Eur J Pediatr 151:154-9, 1992.

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

McCandless DW. Octanoic acid-induced coma and reticular formation energy metabolism. Brain Res 335:131-7, 1985.

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

Mirandola SR, Melo DR, Schuck PF, Ferreira GC, Wajner M, Castilho RF. Methylmalonate inhibits succinate-supported oxygen consumption by interfering with mitochondrial succinate uptake. J Inherit Metab Dis 31:44-54, 2008.

Moshal KS, Metreveli N, Frank I, Tyagi SC. Mitochondrial MMP activation, dysfunction and arrhythmogenesis in hyperhomocysteinemia. Curr Vasc Pharmacol 6:84-92, 2008.

Nelson DL, COX MM. Lehninger Principles of biochemistry. 3ª ed. New York: Worth Publishers, 623-721, 2000.

Nicholls D, Akerman K. Mitochondrial calcium transport. Biochim Biophys Acta 683:57-88, 1982.

Nicholls DG, Ferguson SJ. The Chemiostatic proton circuit In: Bioenergetics 3 (Nicholls DG, Ferguson SJ, eds), 57-87. Academic Press, London, 2002.

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

Olson JE, Holtzman D, Sankar R, Lawson C, Rosenberg R. Octanoic acid inhibits astrocyte volume control: implications for cerebral edema in Reye's syndrome. J Neurochem 52:1197-202, 1989.

Onkenhout W, Venizelos V, van der Poel PFH, Van der Heuvel MPM, Poorthuis BJHM. Identification and quantification of intermediates of unsaturated fatty acid metabolism in plasma of patients with fatty acid oxidation disorders. Clin Chem 41:1467–74, 1995.

Reddy PH, Beal MF. Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease.Trends Mol Med 14:45-53, 2008.

Regenold WT, Phatak P, Marano CM, Sassan A, Conley RR, Kling MA. Elevated cerebrospinal fluid lactate concentrations in patients with bipolar disorder and schizophrenia: implications for the mitochondrial dysfunction hypothesis. Biol Psychiatry 65:489-494, 2009.

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

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

Ribeiro CA, Balestro F, Grando V, Wajner M. Isovaleric acid reduces Na+, K+-ATPase activity in synaptic membranes from cerebral cortex of young rats. Cell Mol Neurobiol 27:529-40, 2007.

Ribeiro CA, Sgaravatti AM, Rosa RB, Schuck PF, Grando V, Schmidt AL, Ferreira GC, Perry ML, Dutra-Filho CS, Wajner M. Inhibition of brain energy metabolism by the branched-chain amino acids accumulating in maple syrup urine disease. Neurochem Res 33:114-24, 2008.

Rinaldo P, Matern D, Bennett MJ. Fatty acid oxidation disorders. Ann Rev Physiol 64:477-502, 2002.

Rinaldo P, Raymond K, Al-Odaib A, Bennett M. Clinical and biochemical features of fatty acid oxidation disorders. Curr Opin Pediatr 10:615–621, 1998.

Roe CR, Ding J. Mitochondrial fatty acid oxidation disorders. In: The Metabolic and Molecular Bases of Inherited Disease (Scriver CR, Beaudet AL, Sly WS, Valle D, eds), 1909–1963, New York: McGraw-Hill, New York, 2001.

Rose CD, Henneberry RC. Etiology of the neurodegenerative disorders: a critical analysis. Neurobiol Aging 15:233-4, 1994.

Ruitenbeek W, Poels PJ, Turnbull DM, Garavaglia B, Chalmers RA, Taylor RW & Gabreels FJ. Rhabdomyolysis and acute encephalopathy in late onset medium chain acyl-CoA dehydrogenase deficiency. J Neurol Neurosurg Psychiatry 58:209-214, 1995.

Samartsev VN, Simonyan RA, Markova OV, Mokhova EN, Skulachev VP. Comparative study on uncoupling effects of laurate and lauryl sulfate on rat liver and skeletal muscle mitochondria. Biochim Biophys Acta 145:179-190, 2000.

Sasaki S, Horie Y, Iwata M. Mitochondrial alterations in dorsal root ganglion cells in sporadic amyotrophic lateral sclerosis. Acta Neuropathol 114:633-9, 2007.

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

Schapira AH, Mann VM, Cooper JM, Dexter D, Daniel SE, Jenner P, Clark JB, Marsden CD. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease, J Neurochem 55:2142– 2145, 1990a.

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

Scholz R, Schwabe U, Soboll S. Influence of fatty acids on energy metabolism. 1. Stimulation of oxygen consumption, ketogenesis and CO_2 production following addition of octanoate and oleate in perfused rat liver. Eur J Biochem 141:223-230, 1984.

Schuck PF, Leipnitz G, Ribeiro CA, Dalcin KB, Assis DR, Barschak AG, Pulrolnik V, Wannmacher CM, Wyse AT, Wajner M. Inhibition of creatine kinase activity *in vitro* by ethylmalonic acid in cerebral cortex of young rats. Neurochem Res 27:1633-9, 2002.

Scriver CR, Beaudet AL, Sly WS, Valle D. (Eds). The Metabolic and Molecular Bases of Inherited Disease. 8ª edition. New York, McGraw-Hill. 3-45, 2001.

Shao L, Martin MV, Watson SJ, Schatzberg A, Akil H, Myers RM, Jones EG, Bunney WE, Vawter MP. Mitochondrial involvement in psychiatric disorders. Ann Med 40:281-295, 2008.

Sitta A, Barschak AG, Deon M, Terroso T, Pires R, Giugliani R, Dutra-Filho CS, Wajner M, Vargas CR. Investigation of oxidative stress parameters in treated phenylketonuric patients.Metab Brain Dis 21:287-96, 2006.

Skulachev VP. Uncoupling: new approaches to an old problem of bioenergetics. Biochim Biophys Acta 1363:100-124, 1998.

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

Smith CM, Marks AD, Lieberman MA. Marks' Basic Medical Biochemistry: A Clinical Approach, 2ª ed. Baltimore: Lippincott Williams & Wilkins, 2005.

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

Trauner DA, Huttenlocher PR. Short chain fatty acid-induced central hyperventilation in rabbits. Neurology 28:940-4, 1978.

Trauner DA. Pathologic changes in a rabbit model of Reye's syndrome. Pediatr Res 16:950-3, 1982.

Trauner DA. Regional cerebral $Na^{+}K^{+}$-ATPase activity following octanoate administration. Pediatr Res 14:844-5, 1980.

Tserng KY, Jin SJ, Hoppel CL. Spiropentaneacetic acid as a specific inhibitor of medium-chain acyl-CoA dehydrogenase. Biochemistry 30:10755-60, 1991.

Tyni T, Paetau A, Strauss AW, Middleton B, Kivelä T. Mitochondrial fatty acid beta-oxidation in the human eye and brain: implications for the retinopathy of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency. Pediatr Res 56:744-50, 2004.

Viegas CM, da Costa Ferreira G, Schuck PF, Tonin AM, Zanatta A, de Souza Wyse AT, Dutra-Filho CS, Wannmacher CM, Wajner M. Evidence that 3-hydroxyisobutyric acid inhibits key enzymes of energy metabolism in cerebral cortex of young rats. Int J Dev Neurosci 26:293-9, 2008.

Waddell L, Wiley V, Carpenter K, Bennetts B, Angel L, Andresen BS, Wilcken B. Medium-chain acyl-CoA dehydrogenase deficiency: genotype-biochemical phenotype correlations. Mol Genet Metab 87:32-9, 2006.

Walker V. Inherited organic acid disorders. In: Clayton BE, Round JM, eds. Clinical Biochemistry and the Sick Child. 131–43, Oxford: Blackwell, 1994.

Wang JF. Defects of mitochondrial electron transport chain in bipolar disorder: implications for mood-stabilizing treatment. Can J Psychiatry 52:753-762, 2007.

Wilcken B, Haas M, Joy P, Wiley V, Chaplin M, Black C, Fletcher J, McGill J, Boneh A. Outcome of neonatal screening for medium-chain acyl-CoA dehydrogenase deficiency in Australia: a cohort study. Lancet 369:37-42, 2007.

Zugno AI, Scherer EB, Schuck PF, Oliveira DL, Wofchuk S, Wannmacher CM, Wajner M, Wyse AT. Intrastriatal administration of guanidinoacetate inhibits Na^+, K^+-ATPase and creatine kinase activities in rat striatum. Metab Brain Dis 21:41-50, 2006.

Zugno AI, Stefanello FM, Scherer EB, Mattos C, Pederzolli CD, Andrade VM, Wannmacher CM, Wajner M, Dutra-Filho CS, Wyse AT. Guanidinoacetate decreases antioxidant defenses and total protein sulfhydryl content in striatum of rats. Neurochem Res 33:1804-10, 2008.