UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS –

BIOQUÍMICA

EFEITOS DE ÁCIDOS GRAXOS HIDROXILADOS DE CADEIA LONGA ACUMULADOS NAS DEFICIÊNCIAS DA 3-HIDROXIACIL-COA DESIDROGENASE DE CADEIA LONGA E DA PROTEÍNA TRIFUNCIONAL MITOCONDRIAL SOBRE A HOMEOSTASE ENERGÉTICA MITOCONDRIAL NOS MÚSCULOS CARDÍACO E ESQUELÉTICO DE RATOS JOVENS

CRISTIANE CECATTO

ORIENTADOR: Prof. Dr. MOACIR WAJNER

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul como requisito parcial à obtenção do grau de Mestre em Bioquímica

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SUMÁRIO

RESUMO

As deficiências da 3-hidroxiacil-CoA desidrogenase de cadeia longa (LCHAD) e da proteína trifuncional mitocondrial (MTP) são defeitos hereditários na oxidação de ácidos graxos. Os pacientes apresentam acúmulo de ácidos graxos hidroxilados de cadeia longa (LCHFA), especialmente os ácidos 3-hidroxitetradecanoico (3HTA) e 3-hidroxipalmítico (3HPA), no sangue e outros tecidos. A sintomatologia é bastante variada, incluindo cardiomiopatia severa e sintomas musculares como fraqueza, dor e episódios recorrentes de rabdomiólise, assim como hepatopatia, retinopatia, hipotonia, neuropatia periférica, atraso no desenvolvimento e na fala, podendo levar a morte ainda na infância. Considerando que os mecanismos patogênicos do dano aos tecidos musculares cardíaco e esquelético apresentados por esses pacientes ainda não estão esclarecidos, o presente trabalho teve como objetivo investigar os efeitos *in vitro* do 3HTA e 3HPA sobre importantes parâmetros da bioenergética mitocondrial, tais como os parâmetros respiratórios estado 3, estado 4, razão de controle respiratório (RCR) e estado desacoplado, bem como o potencial de membrana ($\Delta\Psi m$), o inchamento, a liberação de citocromo *c*, a capacidade de retenção de Ca^{2+} e o conteúdo do NAD(P)H em mitocôndrias isoladas de músculo cardíaco e esquelético de ratos jovens. Inicialmente, observamos que o 3HTA e 3HPA em baixas concentrações (10-30 µM) aumentaram o estado 4 e diminuíram o RCR em mitocôndrias de músculo esquelético, indicando um efeito desacoplador. Quando em concentrações mais elevadas (50-100 µM), esses ácidos graxos diminuíram o estado 4, o estado 3 e o estado desacoplado da respiração mitocondrial, característicos de inibidores metabólicos. Ainda observamos que o 3HPA foi capaz de produzir efeitos semelhantes sobre a respiração mitocondrial em fibras musculares permeabilizadas, validando os resultados obtidos em mitocôndrias isoladas. Além disso, demonstramos que o 3HPA e o 3HTA (30 µM) diminuíram marcadamente o $\Delta\Psi m$, o conteúdo de NAD(P)H, a capacidade de retenção de Ca^{2+} e a produção de ATP, além de induzirem inchamento, em mitocôndrias obtidas de ambos os tecidos e suplementadas com Ca^{2+}. Esses efeitos foram prevenidos por ciclosporina A e ADP, assim como pelo rutênio vermelho, indicando o envolvimento do PTP e

do Ca^{2+}, respectivamente. O fato de termos verificado que o 3HPA aumentou marcadamente a fluidez das membranas mitocondriais em músculo esquelético indica que esse mecanismo pode estar envolvido no prejuízo da homeostase mitocondrial causado por esses compostos. Finalmente, verificamos que o análogo dicarboxílico do 3HTA, o ácido 3-hidroxitetradecanodioico, que também se acumula nos pacientes, não alterou os parâmetros avaliados, indicando uma ação seletiva para os ácidos monocarboxílicos. Analisando nossos resultados em conjunto, demonstramos que os principais LCHFA acumulados nas deficiências da LCHAD e MTP prejudicam a homeostase mitocondrial em músculo cardíaco e esquelético. Presumimos que esse mecanismo possa explicar, pelo menos em parte, a cardiomiopatia severa, os sintomas e alterações musculares que acometem os pacientes afetados.

ABSTRACT

Long-chain 3-hydroxy-acyl-CoA dehydrogenase (LCHAD) and mitochondrial trifunctional protein (MTP) deficiencies are inborn errors of fatty acid oxidation. Affected patients present accumulation of long-chain hydroxylated fatty acids (LCHFA), particularly 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids, in blood and other tissues. The symptomatology is varied, including severe cardiomyopathy and muscular symptoms such as weakness, muscle pain and recurrent episodes of rhabdomyolysis, as well as hepatopathy, retinopathy, hypotonia, peripheral neuropathy, speech and development delay, leading to premature death. Considering that the pathogenesis of cardiac and skeletal muscle damage presented by the patients are still not established, the aim of the present work was to investigate the *in vitro* effects of 3HTA and 3HPA on important parameters of mitochondrial bioenergetics, namely the respiratory parameters state 3, state 4, respiratory control ratio (RCR) and uncoupled respiration, as well as mitochondrial membrane potential ($\Delta\Psi$m), swelling, Ca^{2+} retention capacity and NAD(P)H redox state in cardiac and skeletal muscle mitochondria isolated from young rats. Initially, we observed that 3HTA and 3HPA at lower concentrations (10-30 µM) increased state 4 and decreased RCR in skeletal muscle mitochondria, indicating an uncoupling effect. At higher concentrations (50-100 µM), these fatty acids decreased state 4, state 3 and uncoupled respiration, suggesting metabolic inhibition. Furthermore, we observed that 3HPA was capable to provoke similar effects on mitochondrial respiration in permeabilized skeletal muscle fibers, validating the results obtained in isolated mitochondria. We also demonstrated that 3HPA and 3HTA (30 µM) strongly decreased the $\Delta\Psi$m, NAD(P)H content, Ca^{2+} retention capacity and ATP production, besides inducing swelling, in mitochondria obtained from both tissues and supplemented with Ca^{2+}. These effects were prevented by cyclosporin A and ADP, as well as by ruthenium red, indicating the involvement of mitochondrial permeability transition and Ca^{2+}, respectively. The fact that 3HPA strongly increased the mitochondrial membrane fluidity in skeletal muscle indicates that this mechanism may be involved in the mitochondrial bioenergetics impairment caused by these compounds. Finally,

we verified that the 3HTA dicarboxylic analogue, 3-hydroxytetradecanodioic acid, which also accumulates in the affected patients, did not alter the tested parameters, indicating a selective action of the monocarboxylic acids. Taken together, we demonstrated that the major LCHFA accumulated in LCHAD and MTP deficiencies impair mitochondrial homeostasis in cardiac and skeletal muscle. We presume that these mechanisms may explain, at least in part, the severe cardiomyopathy, symptomatology and muscle alterations characteristics of the patients affected by these disorders.

LISTA DE ABREVIATURAS

3HPA – ácido 3-hidroxipalmítico

3HTA – ácido 3-hidroxitetradecanóico

3HTDA – ácido 3-hidróxitetradecanodióico

ΔΨm – potencial de membrana mitocondrial

Alm – alameticina

ADP – adenosina difosfato

AFLP – fígado gorduroso agudo da gravidez

ANT – translocador de nucleotídeos de adenina

ATC – atractilosídeo

ATP – adenosina trifosfato

CAC – ciclo do ácido cítrico

CCCP – cianeto de carbonila de meta-clorofenil-hidrazona

CoA – coenzima A

CoQ – coenzima Q

CPT – carnitina palmitoil transferase

CsA – ciclosporina A

CypD – ciclofilina D

FADH$_2$ – flavina adenina dinucleotídeo

GDP – guanosina difosfato

HELLP – hemólise, aumento das enzimas hepáticas e baixos níveis de plaquetas

KT - cetotiolase

LCAD – acil-CoA desidrogenase de cadeia longa

LCEH – 2-enoil-CoA hidratase de cadeia longa

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

LCHFA – ácidos graxos hidroxilados de cadeia longa

LCKT – 3-cetoacil-CoA tiolase de cadeia longa

MCAD – acil-CoA desidrogenase de cadeia média

MCT – triglicerídeos de cadeia média

MCU – sistema uniporte de Ca^{2+}

mHCX – trocador H^+/ Ca^{2+}

mNCX – trocador Na^+/ Ca^{2+}

MTP – proteína trifuncional mitocondrial

NAD^+ – nicotinamida adenina dinucleotídeo

NADH – nicotinamida adenina dinucleotídeo reduzida

$NADP^+$ – nicotinamida adenina dinucleotídeo fosfato

NADPH – nicotinamida adenina dinucleotídeo fosfato reduzida

NMDA – N-metil-D-aspartato

Pi – fosfato inorgânico

PTP – poro de transição de permeabilidade

RCR – razão de controle respiratório

RR – rutênio vermelho

SCAD – acil-CoA desidrogenase de cadeia curta

SCEH – enoil-CoA hidratase de cadeia curta

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

UCP – proteínas desacopladoras

VDAC – canal de ânions voltagem-dependente

VLCAD – acil-CoA desidrogenase de cadeia muito longa

Introdução e Objetivos

1. INTRODUÇÃO

1.1. ERROS INATOS DO METABOLISMO

Os erros inatos do metabolismo são doenças genéticas causadas pela deficiência de uma proteína, geralmente uma enzima, levando ao bloqueio de uma rota do metabolismo celular. Esse bloqueio, leva ao acúmulo do substrato da via em questão e à falta dos produtos. O substrato acumulado é levado à metabolização por rotas alternativas, gerando compostos que são potencialmente tóxicos (Scriver 2001) (Figura 1).
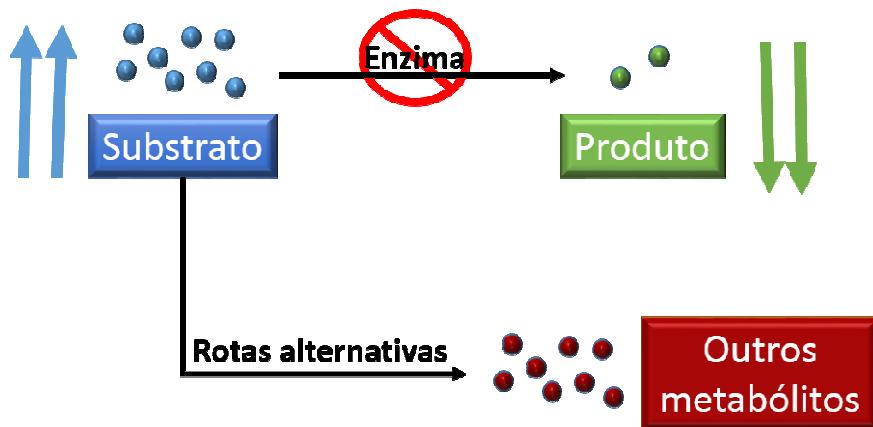
Figura 1. Esquema geral de um bloqueio enzimático que ocorre em erros inatos do metabolismo.

Até o momento, foram descritos mais de 600 erros inatos do metabolismo com o defeito bioquímica caracterizado, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver 2001). Embora individualmente raras essas doenças afetam aproximadamente 1 a cada 4000 recém-nascidos vivos (Ginocchio and Brunetti-Pierri 2015).

1.2. OXIDAÇÃO DE ÁCIDOS GRAXOS

A oxidação de ácidos graxos a acetil-CoA é uma importante rota metabólica para a produção de energia em muitos tecidos. No coração e no fígado, é responsável pela geração de aproximadamente 80% da energia necessária em condições fisiológicas. Também é através dessa via que ocorre a formação de corpos cetônicos no fígado que são exportados para todos os

tecidos, incluindo o cérebro transformando-se em importante fonte de energia em condições de falta de glicose, como por exemplo, o jejum (Nelson and Cox 2013).

Os ácidos graxos de cadeia longa presentes no plasma entram na célula com o auxílio de proteínas de membrana transportadoras de ácidos graxos, que possuem também atividade de acil-CoA sintetase, levando a rápida formação de acil-CoA graxos após a entrada na célula (Houten and Wanders 2010). Na forma de acil-CoA graxos, eles podem ser utilizados para formação de fosfolipídeos, triglicerídeos e ésteres de colesterol ou entrar na mitocôndria para sofrerem a β-oxidação. Como a membrana mitocondrial é impermeável a acil-CoA graxos, faz-se necessário a conjugação com a carnitina através da carnitina palmitoil transferase I (CPT I) da membrana externa da mitocôndria, onde são convertidos a acil-carnitinas que podem atravessar a membrana interna da mitocôndria através do transportador de carnitina/acilcarnitina (CT). Uma vez na matriz mitocondrial, as acilcarnitinas são convertidas a acil-CoA com o auxílio da carnitina palmitoil transferase II (CPT II), regenerando a carnitina (Roe and Ding 2014).

Os acil-CoA graxos passam pela espiral da β-oxidação liberando um acetil-CoA, um NADH e um $FADH_2$ a cada ciclo (Figura 2). Esse acetil-CoA geralmente é oxidado no ciclo do ácido tricarboxílico (CAC) a CO_2 e água em tecidos como coração e músculo esquelético. No fígado, é convertida a corpos cetônicos que são exportados para vários tecidos, tais como o cérebro e os músculos cardíaco e esquelético em situações de estresse catabólico, como inflamação, infecções e jejum prolongado. O NADH e $FADH_2$ atuam como equivalentes reduzidos para a cadeia transportadora de elétrons.

Cada ciclo na espiral da β-oxidação é mediado por quatro reações enzimáticas: acil-CoA desidrogenase, 2-enoil-CoA hidratase, L-3-hidroxiacil-CoA desidrogenase e 3-cetoacil-CoA tiolase, sendo que cada grupo de ácidos graxos (cadeia curta ou cadeia longa) tem enzimas específicas para realizar sua oxidação.

Foi descrita uma proteína trifuncional mitocondrial (MTP) acoplada à membrana interna da mitocôndria constituída de quatro subunidades α e quatro subunidades β (Carpenter, Pollitt et al. 1992). As subunidades α contêm as

atividades das enzimas 2-enoil-CoA hidratase de cadeia longa (LCEH), 3-hidroxiacil-CoA desidrogenase de cadeia longa (LCHAD) e as subunidades β contém a atividade da 3-cetoacil-CoA tiolase de cadeia longa (LCKT) (L, Ruiter et al. 1996). Após algumas voltas realizadas pela MTP, a acil-CoA com a cadeia de carbonos diminuída, passa a ser metabolizada na matriz mitocondrial por enzimas com afinidade aos ácidos graxos de menor número de carbonos.
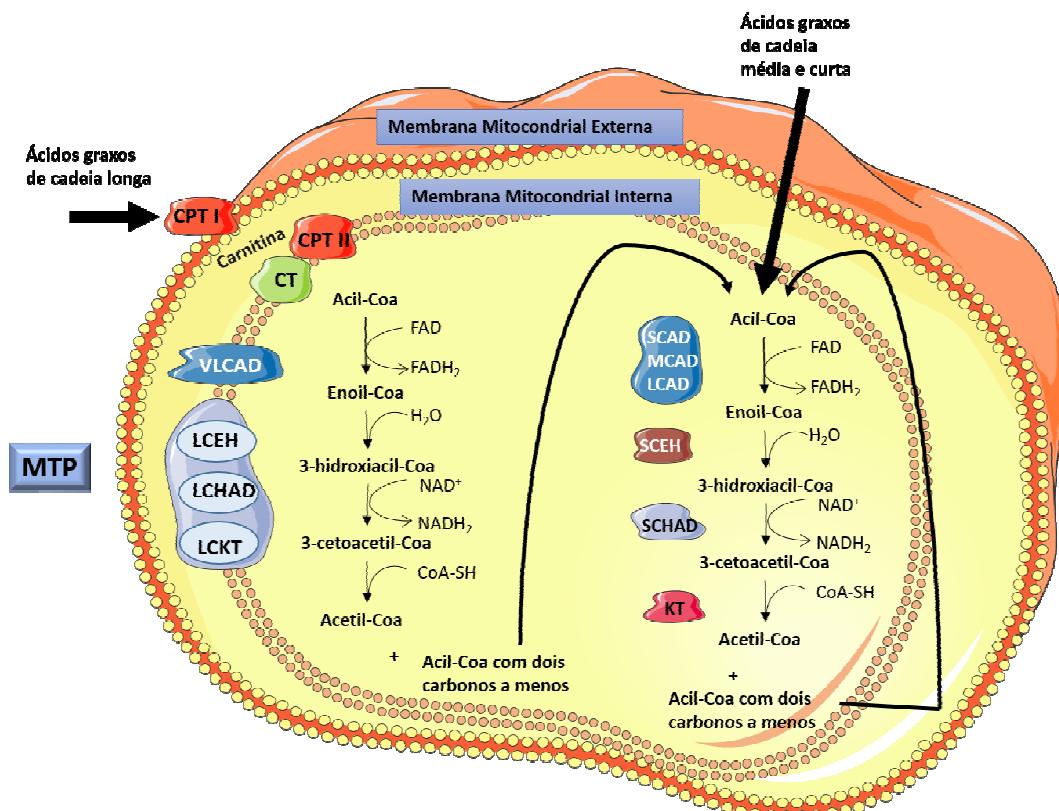
Figura 2. Esquema da β-oxidação de ácidos graxos mitocondrial. Carnitina palmitoil transferases 1 (CPT1) e 2 (CPT2); carnitina-acilcarnitina translocase (CT); acilas-CoA desidrogenase de cadeia muito longa (VLCAD); proteína trifuncional mitocondrial (MTP); 2-enoil-CoA hidratase de cadeia longa (LCEH); 3-hidroxiacil-CoA desidrogenase de cadeia longa (LCHAD); 3-cetoacil-CoA tiolase de cadeia longa (LCKT); acil-CoA desidrogenase de cadeia longa (LCAD); acilas-CoA desidrogenase de cadeia média (MCAD); acilas-CoA desidrogenase de cadeia curta (SCAD); enoil-CoA hidratase de cadeia curta (SCEH); 3-hidroxiacil-CoA desidrogenase de cadeia curta (SCHAD); cetotiolase (KT).

1.3. DEFEITOS ENZIMÁTICOS NA OXIDAÇÃO DE ÁCIDOS GRAXOS

1.3.1. Deficiência da 3-hidroxiacil-CoA desidrogenase de cadeia longa (LCHAD)

Os pacientes acometidos pela deficiência da LCHAD geralmente apresentam inicialmente hipoglicemia hipocetótica e letargia após períodos de jejum acompanhados de algum tipo de infecção, que pode levar a morte nos 3

primeiros meses de vida (den Boer, Wanders et al. 2002; Haglind, Stenlid et al. 2013). Os pacientes afetados também apresentam cardiomiopatia, disfunção severa do fígado com colestase e rabdomiólise. Outros também apresentam complicações a longo prazo, tais como retinopatia pigmentosa e neuropatia periférica (Tyni and Pihko 1999; den Boer, Wanders et al. 2002). Hipotonia, disfunção hepática e cardiomiopatia ocorrem cronicamente, podendo ser causados pelos compostos acumulados (Corr, Creer et al. 1989; den Boer, Wanders et al. 2002).

Em alguns casos, os sintomas são observados no período neonatal e a mãe pode apresentar uma série de alterações bioquímicas e hematológicas (hemólise, aumento das enzimas hepáticas e baixos níveis de plaquetas) durante a gravidez, quadro denominado de síndrome HELLP e AFLP (fígado gorduroso agudo da gravidez). Esse quadro pode acontecer tanto na deficiência da LCHAD quanto na da MTP (Spiekerkoetter, Mueller et al. 2008).

As alterações bioquímicas na deficiência da LCHAD incluem hipoglicemia, acidemia lática e altos níveis séricos de creatina quinase, aspartato aminotransferase e amônia (den Boer, Wanders et al. 2002). Entre os achados patológicos é usualmente encontrado acúmulo de gordura no coração, músculo esquelético e fígado dos pacientes acometidos pela doença (Roe and Ding 2014).

O diagnóstico é feito na urina por cromatografia gasosa acoplada a espectrometria de massas, através da detecção de concentrações elevadas dos 3-hidroxiácidos dicarboxílicos, ou em sangue através de espectrometria de massas em Tandem pelo acúmulo das 3-hidroxiacilcarnitinas (Millington, Terada et al. 1992). Também é possível medir a atividade da LCHAD em alguns tipos celulares, como linfócitos, fibroblastos ou em biópsias de músculo e fígado (Wanders, L et al. 1990; Wanders and Ijlst 1992). A confirmação diagnóstica pode ser feita através da análise mutacional (den Boer, Wanders et al. 2002) por uma mutação comum encontrada nesses pacientes no sítio ativo da subunidade α da proteína trifuncional, onde ocorre a substituição de uma guanina por uma citosina na posição 1528, resultando na substituição de um glutamato por glutamina na posição 510 na região do sítio ativo da proteína (L, Ruiter et al. 1996), acarretando diminuição na atividade da proteína (Hintz,

Matern et al. 2002). Apesar de ser uma mutação comum de ser encontrada, os fenótipos clínicos são bastante heterogêneos (Spiekerkoetter, Khuchua et al. 2004).

1.3.2. Deficiência da Proteína Trifuncional Mitocondrial (MTP)

Menos frequente que a deficiência isolada da LCHAD, a deficiência nas três enzimas do complexo da MTP tem um desfecho mais grave, por geralmente causar morte por cardiomiopatia na infância (Wanders, Vreken et al. 1999; Spiekerkoetter, Khuchua et al. 2004). A deficiência da MTP pode levar a três fenótipos diferentes: letal, hepático e neuromiopático. O fenótipo letal geralmente envolve cardiomiopatia com acidemia lática, sintomas semelhantes aos da síndrome de Reye que se acompanha de hipoglicemia hipocetótica e morte neonatal (Spiekerkoetter, Sun et al. 2003). Também foi reportado cardiomiopatia intrauterina, resultando em morte imediata após o nascimento por insuficiência cardíaca (Spiekerkoetter, Mueller et al. 2008). A forma hepática tende a ser menos severa, iniciando nos primeiros meses de vida com episódios de hipoglicemia hipocetótica e letargia desencadeados por jejum ou infecção. Os pacientes podem ou não apresentar hepatomegalia causada por esteatose hepática (Spiekerkoetter, Mueller et al. 2008). Já a forma neuromiopática tem início mais tarde na vida do paciente, causando neuropatia periférica e mioglobinúria induzida por exercício (Spiekerkoetter, Sun et al. 2003). Assim como na deficiência da LCHAD, os pacientes com a deficiência na MTP podem apresentar neuropatia periférica que é progressiva e irreversível (Spiekerkoetter, Mueller et al. 2008), mas com maior frequência que na deficiência de LCHAD.

Os pacientes também apresentam excreção aumentada de ácidos 3-hidroxilados monocarboxílicos e dicarboxílicos de cadeia longa (Costa, Dorland et al. 1998; Roe and Ding 2014). Não existe nenhuma mutação prevalente, podendo ocorrer tanto nas subunidades α quanto nas β (Spiekerkoetter, Khuchua et al. 2004). Aquelas que acometem as subunidades β geralmente são caracterizadas por fenótipo heterogêneo, predominando, entretanto, sintomas mais brandos (Spiekerkoetter, Sun et al. 2003).

O tratamento para ambas as doenças inclui uma dieta com baixo teor de ácidos graxos de cadeia longa, evitando períodos longos de jejum. Também apresentou benefício o uso de suplementação com MCT (triglicerídeos de cadeia média) (Roe and Ding 2014).

1.3.3. Patogênese das deficiências da LCHAD e MTP

Apesar de existirem poucos estudos na literatura relacionados à patogênese dessas doenças, acredita-se que a alta mortalidade nas deficiências da LCHAD e da MTP ocorre por dois mecanismos: (1) a toxicidade dos ésteres e/ou ácidos de cadeia longa hidroxilada acumulados causando cardiomiopatia e (2) o bloqueio da oxidação de ácidos graxos de cadeia longa e inabilidade de sintetizar compostos cetônicos e ATP (déficit energético) (den Boer, Wanders et al. 2002). Em relação ao primeiro mecanismo, alguns estudos têm demonstrado efeitos tóxicos *in vitro* dos metabólitos acumulados nessas doenças. Foi demonstrado que hidroxiacilas-CoA de cadeia longa inibem a síntese de ATP, o transportador de nucleotídeos de adenina, além de transportadores mitocondriais de ácidos dicarboxílicos, bem como enzimas da cadeia respiratória em preparações mitocondriais de fígado de ratos (Ventura, Ruiter et al. 2005; Ventura, Tavares de Almeida et al. 2007).

Outros estudos demonstraram que os 3-hidroxiácidos de cadeia longa agem como desacopladores da fosforilação oxidativa, inibidores metabólicos e indutores da abertura do PTP em preparações mitocondriais de cérebro de ratos (Tonin, Ferreira et al. 2010; Tonin, Amaral et al. 2014). Também já foi observado que os 3-hidroxiácidos acumulados nas deficiências da MTP e da LCHAD induzem estresse oxidativo *in vitro* em córtex cerebral de ratos jovens (Tonin, Grings et al. 2010). Além disso, foi verificado que os principais ácidos graxos acumulados nessas doenças atuam como desacopladores em mitocôndrias obtidas de coração de ratos (Tonin, Amaral et al. 2013). Em preparações mitocondriais de fígado de ratos também foi observado efeito inibitório dos ácidos graxos de cadeia longa hidroxilados na fosforilação oxidativa e na homeostase mitocondrial, bem como seu envolvimento na abertura do PTP na presença de cálcio, cujos efeitos foram prevenidos por

ciclosporina, ADP (inibidores do PTP) e rutênio vermelho (inibidor da captação de cálcio mitocondrial) (Hickmann, Cecatto et al. 2015).

Esses achados sugerem que a acidemia lática descrita em pacientes afetados por essas doenças possa estar relacionada a uma disfunção mitocondrial associada ao prejuízo na produção de ATP possivelmente devido ao aumento intramitocondrial de acilas-CoA de cadeia longa hidroxilada (Ventura, Ruiter et al. 1998; den Boer, Wanders et al. 2002).

1.4. METABOLISMO ENERGÉTICO

A mitocôndria é a organela responsável pela regeneração de ADP a ATP através da fosforilação oxidativa. Além disso, é nessa organela que há a maior produção de espécies reativas e também de defesas antioxidantes (Cadenas and Davies 2000; Kandola, Bowman et al. 2015). É também responsável pela homeostase do cálcio e participa dos processos de morte celular (Finkel, Menazza et al. 2015; Orrenius, Gogvadze et al. 2015). São envoltas por duas membranas, uma externa permeável a pequenas moléculas e íons, e uma interna impermeável a maioria das moléculas, incluindo ATP, ADP, Pi, piruvato, H^+, NADH, NAD^+, entre outras (Nelson and Cox 2013).

Na fosforilação oxidativa o O_2 é reduzido a H_2O com os elétrons do NADH e $FADH_2$ produzidos no ciclo do ácido cítrico, na oxidação dos ácidos graxos e na utilização dos corpos cetônicos. Os elétrons desses equivalentes reduzidos são passados pelos complexos da cadeia transportadora de elétrons, ou cadeia respiratória até o aceptor final de elétrons: o O_2. 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 (sucinato desidrogenase) reduz a ubiquinona com elétrons do $FADH_2$ provenientes da oxidação do sucinato 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 et al. 1992). Ao serem oxidados, os complexos I, III e IV colocam quatro, quatro e dois prótons no espaço intermembranas, respectivamente, formando um gradiente eletroquímico, uma vez que a matriz mitocondrial é carregada negativamente. Esse gradiente é de fundamental importância para o funcionamento da F_oF_1-ATP sintase que transporta prótons para a matriz gerando uma força próton-motriz necessária para a conversão de ADP + Pi em ATP.

Como a membrana mitocondrial interna é impermeável às moléculas de NADH ou a $FADH_2$, faz-se necessário 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, por meio de moléculas capazes de serem transportadas através da membrana mitocondrial interna. Existem duas lançadeiras para este fim, designadas de lançadeira do glicerol-3-fosfato e lançadeira de malato/aspartato. Uma vez reduzidas 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 and Cox 2013).

1.4.1. Parâmetros respiratórios

O consumo de O_2 permite se avaliar a respiração mitocondrial. Mesmo que essa simples medida determine apenas a velocidade da transferência final de elétrons para o O_2, ou seja, uma única reação, adaptando as condições de incubação, pode-se obter muitas outras informações sobre outros processos mitocondriais, 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 and Ferguson 2002).

Experimentalmente, pode-se dividir a respiração mitocondrial em 5 estágios, embora apenas os parâmetros estados 3 e 4 sejam comumente utilizados. O estado 3 representa o consumo de oxigênio quando as mitocôndrias, em um meio contendo substrato oxidável, são supridas com 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 velocidade da respiração (estado não-fosforilante) (Nicholls and Ferguson 2002) (Figura 3). A transdução de energia entre o gradiente eletroquímico de H^+ e a cadeia respiratória é bem regulada, sendo que um pequeno desequilíbrio termodinâmico entre ambos pode resultar em uma alteração importante no transporte de elétrons pela mesma. Portanto quando a ATP sintase, estimulada pela adição de ADP, dissipa o gradiente de prótons ocorre 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 and Cox 2013). Nesse contexto, o acoplamento da respiração mitocondrial é definido como a capacidade da mitocôndria de gerar ATP quando suprida com 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 and Ferguson 2002).
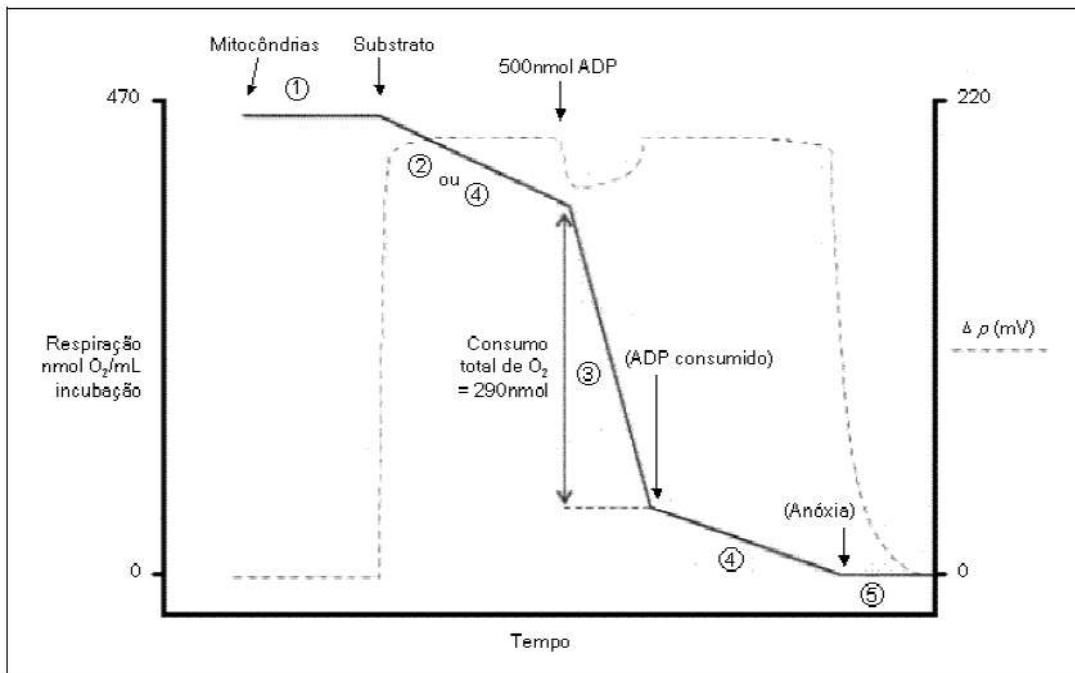
Figura 3. Estados da respiração mitocondrial. (Adaptado de Nicholls e Ferguson, 2002).

Conforme citado anteriormente, a mitocôndria está envolvida em diversos processos celulares, não somente na regeneração do ADP. Acredita-se que todos estes processos estejam interligados e que um desequilíbrio nessas funções possa estar envolvido na fisiopatologia de diversas doenças neurodegenerativas, incluindo as doenças de Alzheimer, Parkinson, Huntington, esclerose lateral amiotrófica (Beal 2007; Sasaki, Horie et al. 2007; Gil and Rego 2008; Reddy and Beal 2008), doenças psiquiátricas, tais como transtorno bipolar, esquizofrenia e depressão (Wang 2007; Ben-Shachar and Karry 2008; Gardner, Salmaso et al. 2008; Shao, Martin et al. 2008; Regenold, Phatak et al. 2009), bem como também de vários erros inatos do metabolismo (Schuck, Leipnitz et al. 2002; Reis de Assis, Maria Rde et al. 2004; Latini, da Silva et al. 2005; Zugno, Scherer et al. 2006; Ferreira, Tonin et al. 2007; Mirandola, Melo et al. 2008; Moshal, Metreveli et al. 2008; Ribeiro, Sgaravatti et al. 2008; Viegas, da Costa Ferreira 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 PTP.

1.4.2. A mitocôndria e o cálcio

Dentre as várias funções desempenhadas pela mitocôndria, uma das mais importantes é manter a homeostase celular de Ca^{2+}, que é responsável por coordenar um vasto repertório de processos na célula. Essa organela tem a capacidade de captar o Ca^{2+} presente no citosol ou liberar seu estoque para a célula, mantendo a concentração intracelular desse cátion em condições ótimas para o funcionamento celular (Rizzuto, De Stefani et al. 2012; Figueira, Barros et al. 2013). Na matriz mitocondrial, o Ca^{2+} é capaz de formar precipitados inativos com o fosfato, o que faz com que a mitocôndria seja capaz de acumular grandes concentrações de Ca^{2+}, podendo chegar a 300 µM dependendo do tecido (Starkov 2010). O Ca^{2+} necessita de transportadores para ser captado e/ou liberado pela mitocôndria. Para acessar o espaço intermembranas ou ser liberado de volta no citosol, o Ca^{2+} utiliza os canais iônicos dependentes de voltagem (VDAC) localizados na membrana mitocondrial externa. Uma vez no espaço intermembranas, o Ca^{2+} é captado pela mitocôndria por um sistema uniporte de Ca^{2+} (MCU) e liberado da matriz pelos trocadores Na^+/Ca^{2+} (mNCX) e H^+/Ca^{2+} (mHCX) (Baughman, Perocchi et al. 2011; De Stefani, Raffaello et al. 2011; Bernardi and von Stockum 2012; Rizzuto, De Stefani et al. 2012; Pan, Liu et al. 2013; Marchi and Pinton 2014; Pendin, Greotti et al. 2014) (Figura 4).
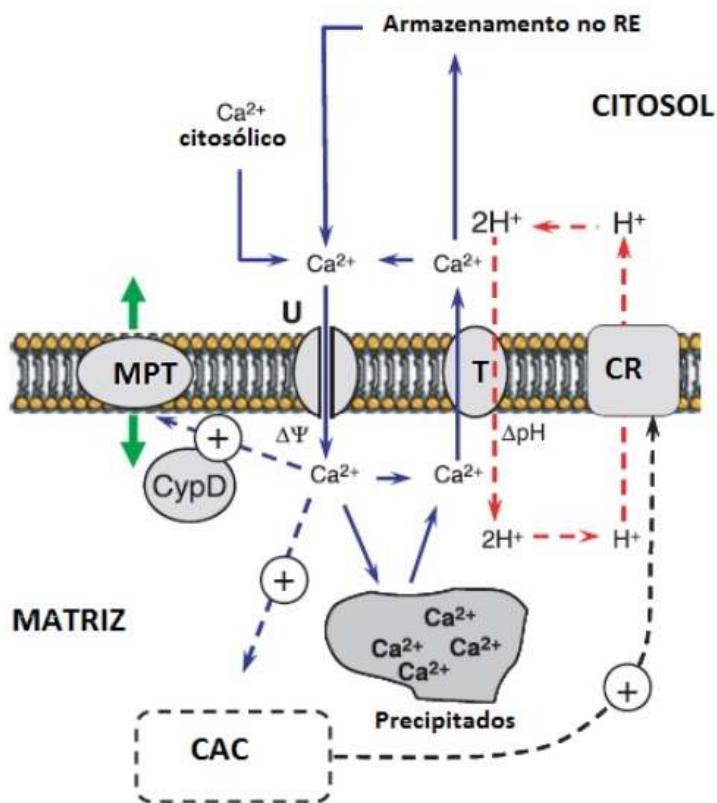
Figura 4. Homeostase mitocondrial de Ca^{2+}. As mitocôndrias acumulam Ca^{2+} exógeno por meio do transportador eletrogênico (uniporte de Ca^{2+}, U) que facilita o transporte de Ca^{2+} através da membrana mitocondrial interna para a matriz. O transporte é acoplado ao acúmulo simultâneo de fosfato inorgânico (não mostrado). Dentro da matriz, o Ca^{2+} e o fosfato acumulados são estocados na forma de precipitados inativos osmoticamente, e eventualmente liberados de volta no citosol através do trocador Ca^{2+}/Na^+ (não mostrado) e/ou $Ca^{2+}/2H^+$, que também estão localizados na membrana mitocondrial interna. O processo de captação de Ca^{2+} é direcionado pelo potencial de membrana mitocondrial; o processo de liberação de Ca^{2+} é direcionado pelo gradiente de pH, no caso do trocador $Ca^{2+}/2H^+$. Elevadas concentrações de Ca^{2+} intramitocondrial podem estimular as atividades das enzimas do ciclo do ácido cítrico (CAC), desse modo impulsionando a produção de energia na mitocôndria. Quando o acúmulo de Ca^{2+} ultrapassa certo limiar, desencadeia-se abertura do PTP, a qual também é modulada pela proteína localizada na matriz ciclofilina D (CypD). T: trocador; CR: cadeia respiratória (Adaptado de Starkov, 2010).

Em várias situações, a mitocôndria se torna a principal organela responsável por captar o excesso de Ca^{2+} citosólico, mantendo os níveis intracelulares desse cátion e o funcionamento celular (Rizzuto, De Stefani et al. 2012; Figueira, Barros et al. 2013). Por outro lado, uma elevada captação mitocondrial de Ca^{2+} além da sua capacidade pode levar a uma condição conhecida como transição de permeabilidade, resultado da abertura de um poro na membrana mitocondrial interna (Zoratti and Szabo 1995; Adam-Vizi and Starkov 2010; Starkov 2010), que está envolvida na neurotoxicidade

induzida por NMDA (Budd and Nicholls 1996; Castilho, Hansson et al. 1998; Stout, Raphael et al. 1998; Pivovarova and Andrews 2010). O PTP mitocondrial é formado por proteínas presentes na matriz mitocondrial ou na membrana mitocondrial interna e possui 2-3 nm de diâmetro. As proteínas que formam o PTP ainda não são muito bem conhecidas, mas devem apresentar três características principais: capaz de se ligar a membrana interna, abrir um canal de 2-3 nm de diâmetro e ser completamente reversível (transitório) (Starkov 2010). Por outro lado, evidências apontam que a ciclofilina D (Cyp D) é uma componente chave para a ocorrência da transição de permeabilidade (Tanveer, Virji et al. 1996; Baines, Kaiser et al. 2005; Basso, Fante et al. 2005; Bernardi 2013). Além disso, o ADP é um potente inibidor da formação do PTP em organelas isoladas de cérebro, indicando o envolvimento do translocador de nucleotídeos (ANT) na formação do poro (Rottenberg and Marbach 1989; Rizzuto, De Stefani et al. 2012).

A permanente abertura do PTP resulta na liberação de Ca^{2+} para o citosol, inchamento mitocondrial, liberação de fatores apoptóticos intramitocondriais como o citocromo c (Liu, Kim et al. 1996), despolarização mitocondrial, perda de metabólitos (Ca^{2+}, Mg^{2+}, GSH, NADH e NADPH), comprometimento da síntese de ATP e morte celular (apoptose e necrose) (Zoratti and Szabo 1995; Crompton, Virji et al. 1999; Starkov 2010; Rasola and Bernardi 2011; Bernardi and von Stockum 2012; Rizzuto, De Stefani et al. 2012; Figueira, Barros et al. 2013).

Muitos trabalhos *in vitro* e *in vivo* tem relacionado disfunção mitocondrial à indução do PTP na presença de Ca^{2+}, evidenciando alteração nos parâmetros da homeostase bioenergética mitocondrial (Maciel, Kowaltowski et al. 2004; Mirandola, Melo et al. 2010; Cecatto, Amaral et al. 2014; Hickmann, Cecatto et al. 2015). Além disso, o ataque oxidativo por espécies reativas de oxigênio está descrito como um importante mecanismo potencializador da abertura do PTP (Kowaltowski, Castilho et al. 2001; Adam-Vizi and Starkov 2010). Neste contexto, altas concentrações de NADH previnem a abertura do PTP, por promover a redução do $NADP^+$ catalisada pela transidrogenase mitocondrial e consequentemente melhoram a capacidade redox da

mitocôndria (Lehninger, Vercesi et al. 1978; Hoek and Rydstrom 1988; Zago, Castilho et al. 2000).

O PTP é inibido por ciclosporina A (CsA), um conhecido peptídeo que se liga e inibe a proteína Cyp D (Starkov 2010; Bernardi and von Stockum 2012; Rizzuto, De Stefani et al. 2012). Foi demonstrado um efeito protetor dessa droga em modelos de isquemia (Nieminen, Petrie et al. 1996; Schinder, Olson et al. 1996; Uchino, Elmer et al. 1998; Petersen, Castilho et al. 2000), sugerindo a importância do PTP na patogênese do dano isquêmico. Neste particular, o PTP tem demonstrado um importante papel na lesão de isquemia-reperfusão, que ocorre após um dano isquêmico cardíaco, e sua inibição mostrou ser cardioprotetora (Ong, Dongworth et al. 2014; Halestrap and Richardson 2015; Ong, Samangouei et al. 2015).

Observou-se também que o PTP está envolvido no envelhecimento do músculo esquelético (Hepple 2014), além de seu envolvimento na disfunção mitocondrial associada com a resistência à insulina em músculo esquelético de ratos nocaute para a CypD (Taddeo, Laker et al. 2014). Disfunção mitocondrial com abertura do PTP está também envolvida em diversas doenças que afetam o músculo esquelético e os nervos periféricos, como por exemplo em distrofias congênitas pela deficiência de colágeno VI e na distrofia muscular de Duchenne (Katsetos, Koutzaki et al. 2013).

2. OBJETIVOS

2.1. OBJETIVO GERAL

O objetivo geral deste trabalho foi investigar os efeitos *in vitro* dos principais ácidos graxos monocarboxílicos acumulados nas deficiências de LCHAD e MTP sobre importantes parâmetros da homeostase energética em coração e músculo esquelético de ratos jovens, visando uma melhor compreensão dos mecanismos tóxicos desses ácidos graxos sobre estes tecidos.

2.2. OBJETIVOS ESPECÍFICOS

- Investigar o efeito *in vitro* dos ácidos 3-hidroxitetradecanóico (3HTA) e 3-hidroxipalmítico (3HPA) sobre alguns parâmetros importantes da homeostase energética em preparações mitocondriais de músculo cardíaco e esquelético de ratos de 30 dias de vida na presença ou ausência de cálcio.

Analisar parmâmetros respiratórios (estado 3, estado 4, estado desacoplado e RCR), potencial de membrana mitocondrial, inchamento mitocondrial, retenção de cálcio, conteúdo dos equivalentes reduzidos de NADH e NADPH e produção de ATP.

Investigar também os mecanismos envolvidos nas modificações observadas, tais como a influência do cálcio na ação deletéria desses metabólitos, bem como o envolvimento do translocador de nucleotídeos (ANT), da proteína desacopladora (UCP) e do PTP mitocondrial, além de alterações na fluidez das membranas mitocondriais.

Além disso, estudar os efeitos do ácido 3-hidroxitetradecanedióico (3HTDA), análogo dicarboxílico do 3HTA sobre alguns desses parâmetros em preparações mitocondriais de músculo cardíaco e esquelético.

Capítulo I

Deregulation of mitochondrial functions provoked by long-chain fatty acid accumulating in long-chain3-hydroxyacyl-CoA dehydrogenase and mitochondrial permeability transition deficiencies in rat heart – mitochondrial permeability transition pore opening as a potential contributing pathomechanism of cardiac alterations in these disorders

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Deregulation of mitochondrial functions provoked by long-chain fatty acid accumulating in long-chain 3-hydroxyacyl-CoA dehydrogenase and mitochondrial permeability transition deficiencies in rat heart – mitochondrial permeability transition pore opening as a potential contributing pathomechanism of cardiac alterations in these disorders

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Mitochondrial trifunctional protein and long-chain 3-hydroxyacyl-CoA dehydrogenase deficiencies are fatty acid oxidation disorders biochemically characterized by tissue accumulation of long-chain fatty acids and derivatives, including the monocarboxylic long-chain 3-hydroxy fatty acids (LCHFAs) 3-hydroxytetradecanoic acid (3HTA) and 3-hydroxypalmitic acid (3HPA). Patients commonly present severe cardiomyopathy for which the pathogenesis is still poorly established. We investigated the effects of 3HTA and 3HPA, the major metabolites accumulating in these disorders, on important parameters of mitochondrial homeostasis in Ca^{2+}-loaded heart mitochondria. 3HTA and 3HPA significantly decreased mitochondrial membrane potential, the matrix NAD(P)H pool and Ca^{2+} retention capacity, and also induced mitochondrial swelling. These fatty acids also provoked a marked decrease of ATP production reflecting severe energy dysfunction. Furthermore, 3HTA-induced mitochondrial alterations were completely prevented by the classical mitochondrial permeability transition (mPT) inhibitors cyclosporin A and ADP, as well as by ruthenium red, a Ca^{2+} uptake blocker, indicating that LCHFAs induced Ca^{2+}-dependent mPT pore opening. Milder effects only achieved at higher doses of LCHFAs were observed in brain mitochondria, implying a higher vulnerability of heart to these fatty acids. By contrast, 3HTA and docosanoic acids did not change mitochondrial homeostasis, indicating selective effects for monocarboxylic LCHFAs. The present data indicate that the major LCHFAs accumulating in mitochondrial trifunctional protein and long-chain 3-hydroxyacyl-CoA dehydrogenase deficiencies induce mPT pore opening, compromising Ca^{2+} homeostasis and oxidative phosphorylation more intensely in the heart. It is proposed that these pathomechanisms may contribute at least in part to the severe cardiac alterations characteristic of patients affected by these diseases.

Introduction

Fatty acid oxidation is an important source of energy production in many tissues, including the heart [1]. Fatty acid oxidation defects are relatively frequent inherited metabolic disorders characterized biochemically by a high elevation of the concentrations of specific fatty acids involved in the various pathways and, clinically, by variable clinical manifestations, some of them with severe cardiac symptoms and signs [2].

The mitochondrial trifunctional protein (MTP) is an enzyme complex coupled to the inner mitochondrial membrane that catalyses the oxidation of long-chain 3-hydroxy fatty acids (LCHFAs). It is composed of three enzyme activities: long-chain enoyl-CoA hydratase, long-chain ketoacyl-CoA thiolase and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD). Molecular defects in MTP-encoding genes lead to deficiencies of isolated enzymatic activities, more commonly LCHAD deficiency, or of all three enzymatic components characterizing MTP deficiency [3]. Furthermore, only few cases of long-chain ketoacyl-CoA thiolase deficiency have been reported [4,5].

MTP and LCHAD deficiencies are clinically and biochemically undistinguishable, presenting with a multi-organ involvement and elevated levels of long-chain fatty acids and their hydroxylated derivatives (i.e. LCHFAs), including 3-hydroxytetradecanoic acid (3HTA), 3-hydroxypalmitic acid (3HPA) and 3-hydroxytetradecanedioic acid (3HTDA), in tissues and biological fluids [4,6,7]. The early onset severe form of MTP deficiency predominantly manifests as acute cardiac manifestations with life-threatening arrhythmias during metabolic crises and liver dysfunction, whereas the late-onset forms are clinically milder and mainly characterized by neuromyopathic signs [8–11]. Fatal cardiomyopathy also occurs immediately after birth in a considerable number of patients affected by LCHAD deficiency [12,13]. Examination of the heart shows cardiomegaly, poor heart sounds, gallop rhythm, left ventricular hypertrophy, dilatation, poor contractility and pericardial effusion. Patients with LCHAD deficiency also present hepatomegaly, skeletal myopathy with muscle weakness, rhabdomyolysis and increased serum creatine kinase, hypoglycemia and lactic acidosis, whereas others have peripheral neuropathy and retinopathy [12,14]. Neuroradiological imaging reveals bilateral periventricular or focal lesions in the cerebral cortex and brain atrophy [11,15].

The pathogenesis of the tissue damage in these disorders has been long attributed to a blockage of fatty acid oxidation and a low availability of glucose (hypoglycemia) leading to energy deficiency. However, we cannot exclude the possibility that the LCHFAs or their 3-hydroxy-long chain acylcarnitines accumulating in these disorders are toxic to cells [8,16].

Recent studies have demonstrated the induction of oxidative stress in skin fibroblasts from MTP deficient patients [17], in accordance with experimental animal studies revealing that LCHFAs provoke lipid and protein oxidative damage and reduce antioxidant defenses in the rat cerebral cortex *in vitro* [18]. Disturbed oxidative phosphorylation was also shown to be caused by these fatty acids in the brain and heart of adolescent rats [19,20], and this may be associated with a secondary respiratory chain defect observed in the skeletal muscle of a LCHAD deficient patient [21]. These findings, reflecting disturbances of redox and energy mitochondrial homeostasis, may possibly explain the observations of an abnormal mitochondrial morphology with a swollen appearance as obtained in muscle biopsies from LCHAD deficient patients [22]. Another study demonstrated that the LCHFAs induce mitochondrial permeability transition (mPT) in Ca^{2+}-loaded brain mitochondria [23]. However, more work is necessary to clarify in more detail the exact role of LCHFAs on heart bioenergetics because this organ is predominantly affected in the severe forms of LCHAD and MTP deficiencies [24,25].

Therefore, in the present study, we investigated the role of 3HTA and 3HPA, the major LCHFAs accumulating in LCHAD and MTP deficiencies, on a large spectrum of mitochondrial homeostasis measurements, including membrane potential ($\Delta\Psi$m), NAD(P)H content, swelling, Ca^{2+} retention capacity and ATP formation in Ca^{2+}-loaded mitochondrial preparations from the heart of young rats. We also used brain mitochondria aiming to compare the susceptibility of heart and brain to the toxic effects of these hydroxylated fatty acids.

Results

LCHFAs reduce $\Delta\Psi$m in heart and brain mitochondria

We first assessed the effects of 3HTA, 3HPA and 3HTDA at the dose of 30 μM on $\Delta\Psi$m in heart and brain mitochondria loaded with Ca^{2+} using glutamate plus malate as substrates. Docosanoic acid (DCA) was also tested only in heart mitochondria. We confirmed that 3HTA and 3HPA, but not 3HTDA, significantly dissipated $\Delta\Psi$m in both tissues, and that these effects were approximately three-fold stronger in the heart compared to the brain (heart: $F_{4,10} = 181.759$, $P < 0.001$; brain: $F_{38} = 13.313$, $P < 0.01$) (Fig. 1). Fur-

thermore, DCA was unable to alter ΔΨm in the heart (Fig. 1A). When various concentrations of 3HTA were used (Fig. 1C,D), ΔΨm dissipation was more pronounced in the heart and was obtained with lower doses of 3HTA (heart: $F_{3,8} = 58.817$, $P < 0.001$; brain: $F_{3,8} = 12.021$, $P < 0.01$).

LCHFAs decrease matrix NAD(P)H content in heart and brain mitochondria

Next, we investigated the influence of 3HTA, 3HPA and 3HTDA on matrix NAD(P)H content in heart and brain mitochondria supported by glutamate plus malate. It was found that 3HTA (10–30 μM) and 3HPA (30 μM) significantly decreased heart mitochondrial NAD(P)H content in the absence of Ca^{2+}, with a greater reduction observed (five-fold compared to control) after the addition of Ca^{2+} ($F_{3,8} = 22.840$, $P < 0.001$; $F_{3,8} = 12.411$, $P < 0.01$) (Fig. 2A,C). By contrast, NAD(P)H content was not changed by

3HTA and 3HPA in brain mitochondrial preparations devoid of exogenous Ca^{2+}, although it was moderately reduced (3.5-fold) after Ca^{2+} supplementation ($F_{3,8} = 11.190$, $P < 0.01$; $F_{3,6} = 8.701$, $P < 0.01$) (Fig. 2B,D).

LCHFAs induce swelling in heart and brain mitochondria

We also observed that 3HTA and 3HPA at the dose of 30 μM, but not 3HTDA, induced a marked swelling in heart (20-fold) and moderate in brain (five-fold) mitochondria supported by glutamate plus malate ($F_{3,8} = 131.906$, $P < 0.001$; $F_{3,8} = 35.600$, $P < 0.001$) (Fig. 3).

LCHFAs reduce Ca^{2+} retention capacity in heart and brain mitochondria

Next, we tested the effects of 3HTA, 3HPA and 3HTDA on mitochondrial Ca^{2+} retention capacity in
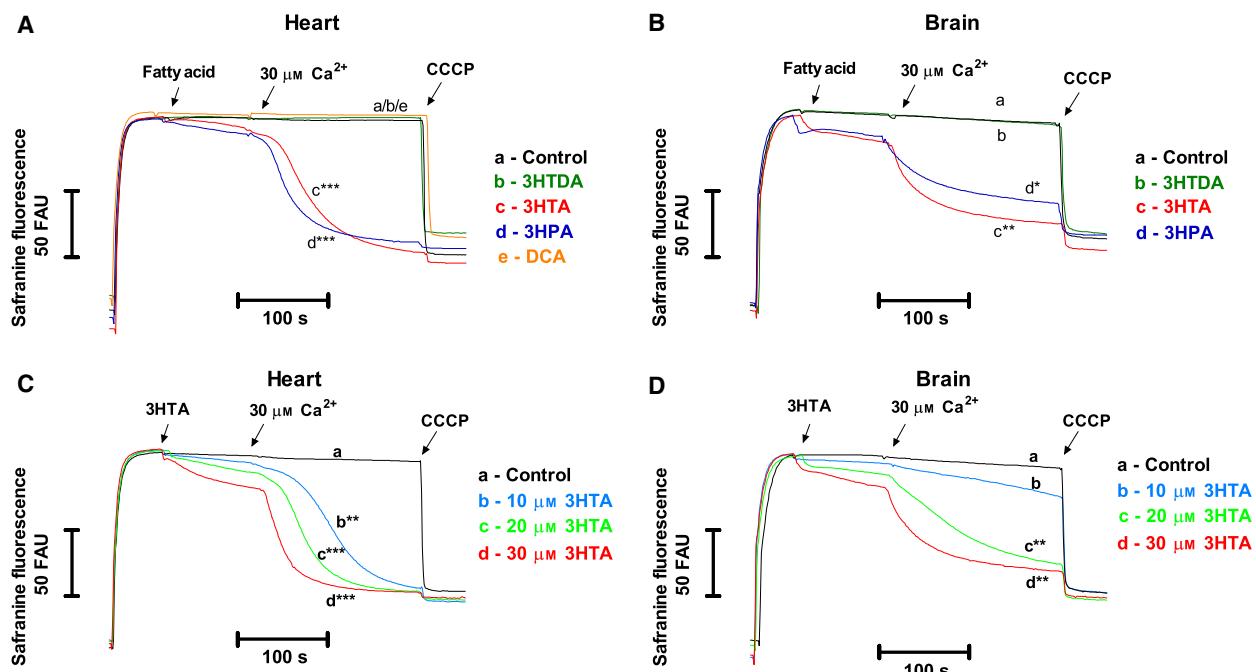
Fig. 1. Effects of 3HTA, 3HPA, 3HTDA and DCA on ΔΨm in Ca^{2+}-loaded mitochondria from the heart (A, C) and brain (B, D). All of the experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein·mL^{-1} for heart and 0.5 mg protein·mL^{-1} for brain) supported by glutamate plus malate. (A, B) 3HTDA, 3HTA, 3HPA and DCA (30 μM, lines b–e) were added 50 s after the beginning of the assay. (C–D) 3HTA (10–30 μM, lines b–d). Controls (line a) were performed in the absence of fatty acids. All panels refer to mitochondrial preparations supplemented by 30 μM Ca^{2+} 150 s after the beginning of the assay, as indicated. CCCP (3 μM) was added at the end of the assays. Fluorescence changes between 150 and 250 s were: (A) 2.23 ± 0.46 (Control), 1.07 ± 0.33 (3HTDA), 72.85 ± 10.33 (3HTA), 66.69 ± 3.03 (3HPA) and 0.68 ± 0.21 (DCA); (B) 4.86 ± 1.59 (Control), 5.71 ± 1.64 (3HTDA), 50.00 ± 16.73 (3HTA) and 28.53 ± 11.45 (3HPA); (C) 2.96 ± 0.23 (Control), 33.63 ± 14.45 (10 μM 3HTA), 75.48 ± 2.78 (20 μM 3HTA) and 68.37 ± 3.56 (30 μM 3HTA); (D) 5.34 ± 2.72 (Control), 18.41 ± 9.68 (10 μM 3HTA), 41.89 ± 11.56 (20 μM 3HTA) and 42.55 ± 10.00 (30 μM 3HTA). Traces are representative of three independent experiments (animals) and are expressed as fluorescence arbitrary units (FAU). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to controls (Duncan's multiple range test).

the heart and brain. DCA was also tested only in heart mitochondria. Both 3HTA and 3HPA compromised mitochondrial Ca^{2+} retention capacity, which was much more evident in the heart, occurring at lower doses (20–30 μM) of 3HTA and 3HPA in this tissue compared to brain mitochondria (60 μM) (Fig. 4). By contrast, 3HTDA and DCA did not alter this important mitochondrial function.

LCHFAs induce mitochondrial permeability transition in heart mitochondria

It was also observed that the reductions of mitochondrial $\Delta\Psi m$ ($F_{3,8} = 221.566$, $P < 0.001$) (Fig. 5A) and NAD(P)H content ($F_{3,8} = 66.949$, $P < 0.001$) (Fig. 5B), as well as the induction of mitochondrial swelling ($F_{3,8} = 37.660$, $P < 0.001$) (Fig. 5C), provoked by 3HTA in Ca^{2+}-loaded heart mitochondrial preparations were completely prevented by ruthenium red

(RR), an inhibitor of mitochondrial Ca^{2+} uptake, and by cyclosporin A (CsA) plus ADP, inhibitors of mPT. Furthermore, CsA completely prevented the strong decrease of Ca^{2+} retention capacity caused by 3HTA (Fig. 5D). The data provide solid evidence that these LCHFAs accumulating most in patients affected by the deficiencies of LCHAD and MTP induce mPT in heart mitochondria.

LCHFAs markedly reduce ATP production in heart mitochondria

We also investigated whether 3HTA and 3HPA could alter ATP production in heart mitochondria. We confirmed that 3HTA (40% inhibition) and more markedly 3HPA (70% inhibition) at the dose of 20 μM decreased ATP formation in mitochondria loaded with Ca^{2+} ([$F_{2,12} = 109.385$, $P < 0.001$] (Fig. 6), probably indicating that the deleterious effects of these LCHFAs
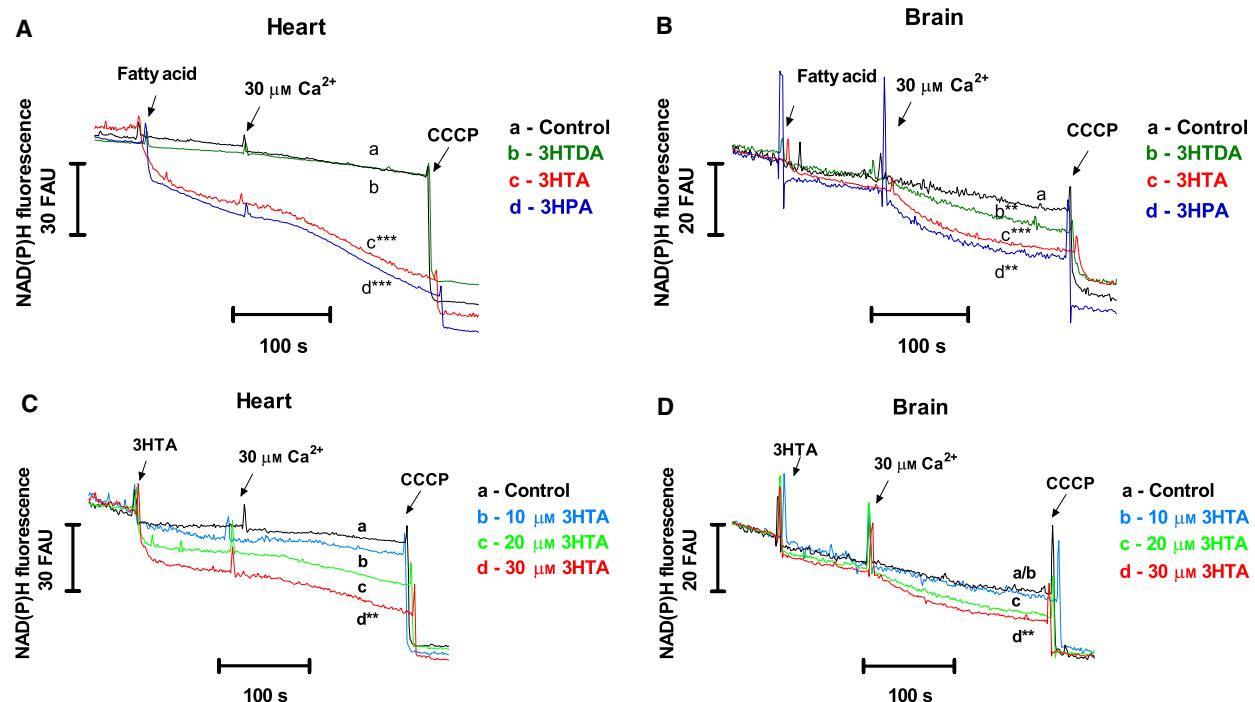
Fig. 2. Effects of 3HTA, 3HPA and 3HTDA on matrix NAD(P)H content in Ca^{2+}-loaded mitochondria from the heart (A, C) and brain (B, D). All of the experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein·mL^{-1} for heart and 0.5 mg protein·mL^{-1} for brain) supported by glutamate plus malate. (A, B) 3HTDA, 3HTA or 3HPA (30 μM, lines b–d) were added 50 s after the beginning of the assay. (C, D) 3HTA (10–30 μM, lines b–d). Controls (line a) were performed in the absence of fatty acids. All panels refer to mitochondrial preparations supplemented by 30 μM Ca^{2+} 150 s after the beginning of the assay, as indicated. CCCP (3 μM) was added at the end of the assays. Fluorescence changes between 150 and 250 s were: (A) 2.62 ± 0.62 (Control), 2.18 ± 1.23 (3HTDA), 12.58 ± 2.16 (3HTA) and 13.82 ± 3.74 (3HPA); (B) 4.22 ± 1.05 (Control), 11.84 ± 3.18 (3HTDA), 14.62 ± 0.74 (3HTA) and 11.73 ± 3.09 (3HPA); (C) 1.91 ± 0.82 (Control), 1.50 ± 0.50 (10 μM 3HTA), 2.93 ± 1.44 (20 μM 3HTA) and 6.17 ± 1.15 (30 μM 3HTA); (D) 4.76 ± 0.50 (Control), 4.57 ± 0.35 (10 μM 3HTA), 8.08 ± 0.86 (20 μM 3HTA) and 17.89 ± 6.11 (30 μM 3HTA). Traces are representative of three independent experiments (animals) and are expressed as fluorescence arbitrary units (FAU). **$P < 0.01$; ***$P < 0.001$ compared to controls (Duncan's multiple range test).
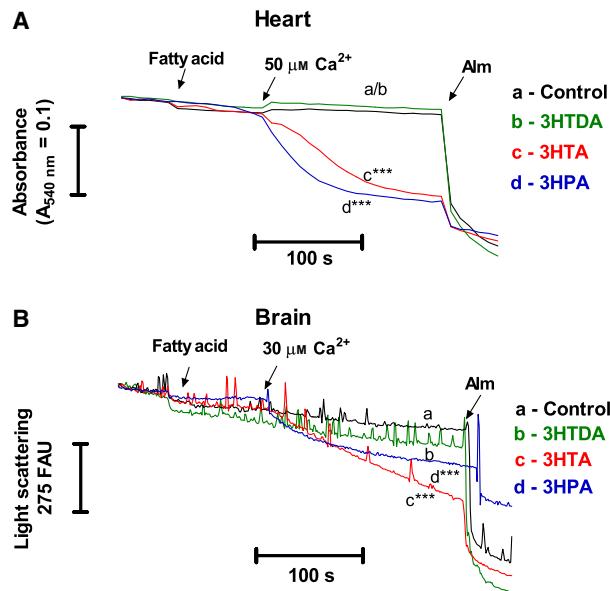
Fig. 3. Effects of 3HTA, 3HPA and 3HTDA on swelling in Ca^{2+}-loaded mitochondria from the heart (A) and brain (B). All of the experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein·mL^{-1} for heart and 0.5 mg protein·mL^{-1} for brain) supported by glutamate plus malate. 3HTDA, 3HTA or 3HPA (30 μM, lines b–d) were added 50 s after the beginning of the assay. Controls (line a) were performed in the absence of fatty acids. All panels refer to mitochondrial preparations supplemented by 40 μM Ca^{2+} 150 s after the beginning of the assay, as indicated. Alm (40 μg·mg protein^{-1}) was added at the end of the measurements. Light scattering changes between 150 and 250 s were: (A) 0.0057 ± 0.0024 (Control), 0.0071 ± 0.0026 (3HTDA), 0.0935 ± 0.0027 (3HTA) and 0.0932 ± 0.0145 (3HPA); (B) 46.33 ± 18.93 (Control), 50.33 ± 9.29 (3HTDA), 191.67 ± 10.12 (3HTA) and 166.33 ± 37.53 (3HPA). Traces are representative of three independent experiments (animals) and are expressed as absorbance (A) or fluorescence arbitrary units (FAU) (B). ***$P < 0.001$ compared to controls (Duncan's multiple range test).

on mitochondrial energy and Ca^{2+} homeostasis compromise energy production.

Discussion

Severe acute cardiac manifestations that may lead to a fatal outcome occur in patients with LCHAD and MTP deficiencies, particularly during crises of metabolic decompensation, which are accompanied by a pronounced increase of the accumulating metabolites [15,24,26]. It should be emphasized that cardiomyopathy is usually not observed between crises and that the severe cardiac phenotype can be avoided by appropriate therapeutic measures. Although the pathogenesis of the cardiac alterations in these diseases is practically

unknown, it has been suggested that accumulation of long-chain acyl-CoA esters and β-oxidation intermediates, including the LCHFAs, may contribute to the clinical phenotypes in long-chain fatty acid oxidation deficient patients [27]. Therefore, considering that energy homeostasis is crucial for normal heart functioning and that fatty acids are the preferential substrates for energy production in myocardium [28], the present study investigated the effects of the major accumulating LCHFAs in MTP and LCHAD deficiencies, namely 3HTA and 3HPA, on important mitochondrial functions, including energy and Ca^{2+} homeostasis in the heart of young rats, aiming to clarify the pathophysiology of the cardiac alterations in these diseases. Mitochondrial functions were also evaluated in brain to compare the magnitude of the effects of these LCHFAs in the heart and brain. Lactic acidemia/lactic aciduria, the inhibition of various complexes of the respiratory chain and mitochondrial morphological abnormalities found in patients affected by these diseases all point to a compromised mitochondrial energy homeostasis [9,21,22,29].

Overall, we demonstrated that 3HTA and 3HPA, at similar or even lower concentrations (10–20 μM) than those found in plasma of LCHAD and MTP deficient patients, significantly impaired various bioenergetics parameters tested, as well as mitochondrial Ca^{2+} retention capacity, inducing mPT opening and drastically reducing energy (ATP) production in Ca^{2+}-loaded mitochondria from the heart. We also confirmed that these LCHFAs changed the same parameters in brain mitochondria, although to a much lower degree and only at higher concentrations (30–60 μM), indicating a greater vulnerability of the heart to the deleterious effects of these accumulating fatty acids.

Thus, 3HTA and 3HPA significantly dissipated ΔΨm in brain and more strongly in heart mitochondria. Furthermore, the addition of Ca^{2+} enhanced mitochondrial membrane potential (ΔΨm) dissipation, which was fully prevented by the classical inhibitor of the mitochondrial Ca^{2+} uniporter, RR [30], indicating that Ca^{2+} is important for this effect. The 3HTA-induced ΔΨm decrease was a result of mPT induction because CsA and ADP normalized ΔΨm. In this context, it is emphasized that CsA is a peptide that inhibits mPT by binding to cyclophilin D, a protein necessary for mPT pore formation [31–34]. Otherwise, ADP is also a potent inhibitor of mPT because it interacts with the adenine nucleotide translocator [35,36].

3HTA and 3HPA also decreased matrix NAD(P)H content and induced swelling in Ca^{2+}-loaded heart
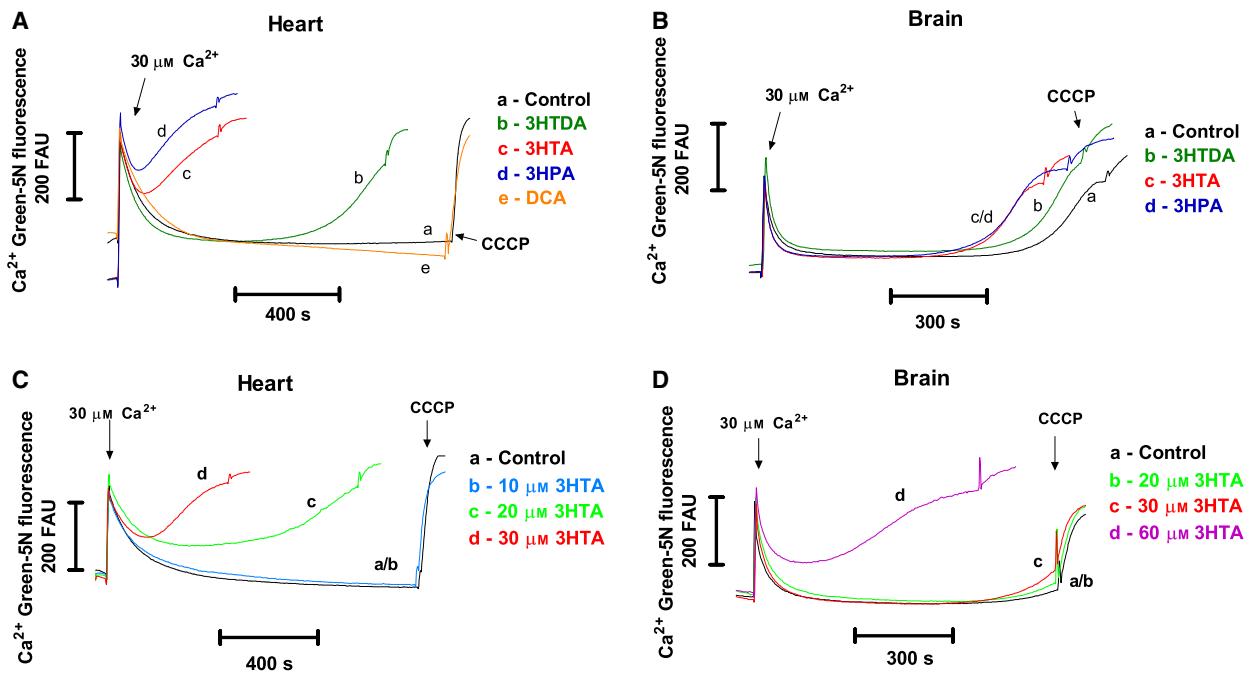
Fig. 4. Effects of 3HTA, 3HPA, 3HTDA and DCA on Ca^{2+} retention capacity in Ca^{2+}-loaded mitochondria from the heart (A, C) and brain (B, D). All experiments were performed in a reaction medium containing mitochondrial preparations (0.2 mg protein·mL^{-1} for heart and 0.5 mg protein·mL^{-1} for brain) supported by glutamate plus malate. (A, B) 3HTDA, 3HTA, 3HPA and DCA (30 μM, lines b–e) were added at the beginning of the assay. (C) 3HTA (10–30 μM, lines b–d). (D) 3HTA (20–60 μM, lines b–d). Controls (line a) were performed in the absence of fatty acids. All panels refer to mitochondrial preparations supplemented by 30 μM Ca^{2+} 60 s after the beginning of the assay, as indicated. CCCP (3 μM) was added at the end of the assays. Traces are representative of three independent experiments (animals) and are expressed as fluorescence arbitrary units (FAU).

mitochondria, which were also totally prevented by RR and CsA plus ADP, reinforcing the role of Ca^{2+} dependent mPT opening in these effects. Noteworthy, mPT activation leads to mitochondrial swelling, loss of mitochondrial elements (Ca^{2+}, Mg^{2+}, glutathione, NADH and NADPH), $\Delta\Psi m$ dissipation and increased NADH oxidation, as well as impairment of oxidative phosphorylation and ATP synthesis [37–39]. These findings are probably related to the marked decrease of ATP formation caused by 3HTA and 3HPA in the heart. On the other hand, we cannot rule out the possibility that the decrease of mitochondrial reducing equivalents and $\Delta\Psi m$ provoked by of 3HTA and 3HPA in the absence of exogenous Ca^{2+} were at least partly a result of the uncoupling behavior of these fatty acids [19,40].

Another novel finding of the present study is that 3HTA and 3HPA strongly reduced the capacity of heart mitochondria to uptake and retain Ca^{2+}, a critical function of this organelle [41–43], which was milder in the brain. Furthermore, this effect was normalized by CsA, indicating that mPT pore opening allows the release of Ca^{2+} from the mitochondrial matrix [44,45].

Because dysregulation of Ca^{2+} homeostasis is associated with disturbance of energy and redox homeostasis [46–50], changes of mitochondrial Ca^{2+} concentrations may also have contributed to the altered parameters of mitochondrial bioenergetics caused by 3HTA and 3HPA. It should be emphasized that intracellular concentrations of Ca^{2+} are critical for the normal functioning of cardiomyocytes. Furthermore, mitochondrial Ca^{2+} homeostasis emerges as an important regulatory mechanism of cardiac physiology that may be affected in pathological conditions [51].

With regard to the mechanisms underlying LCHFA-induced mPT opening in Ca^{2+}-loaded mitochondria, these may be provoked by oxidative stress, as well as by oxidative phosphorylation uncouplers that lead to NADH oxidation and secondarily nonselective permeabilization [52–57]. In this scenario, LCHFAs were demonstrated to provoke oxidative stress and uncouple the oxidative phosphorylation [18,19,40].

Another interesting observation made in the present study is that the deleterious effects were selective for the monocarboxylic LCHFAs because the dicarboxylic 3HTDA, a structurally analogue of 3HTA, did not
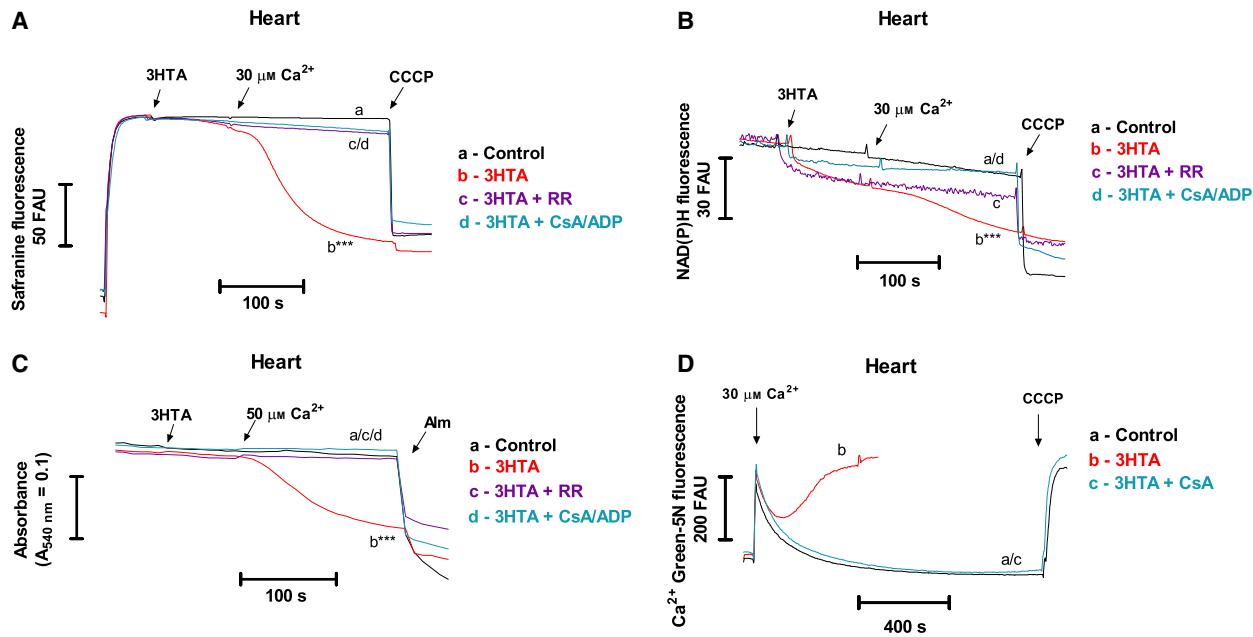
Fig. 5. Effects of 3HTA on $\Delta\Psi$m (A), matrix NAD(P)H content (B), swelling (C) and Ca^{2+} retention capacity (D) in Ca^{2+}-loaded mitochondria from the heart. All experiments were performed in a reaction medium containing heart mitochondrial preparations [0.35 mg protein·mL^{-1} in (A) to (C) and 0.2 mg protein·mL^{-1} in (D)] supported by glutamate plus malate. (A–C) 3HTA (30 μM, lines b–d) was added 50 s after the beginning of the assay. (D) 3HTA (30 μM, lines b–d) was added at the beginning of the assay. Controls (line a) were performed in the absence of fatty acids. Panels refer to mitochondrial preparations supplemented by 30 μM Ca^{2+} (A, B and D) or 40 μM (C) Ca^{2+}. RR (1 μM), CsA (1 μM) and ADP (300 μM) were added at the beginning of the assay, as indicated. CCCP (3 μM) (A, B and D) or Alm (40 μg·mg protein^{-1}) (C) were added at the end of the measurements. Fluorescence and light scattering changes between 150 and 250 s were: (A) 2.23 ± 0.46 (Control), 64.60 ± 6.79 (3HTA), 5.81 ± 0.99 (3HTA + RR) and 4.00 ± 1.57 (3HTA + CsA/ADP); (B) 2.62 ± 0.62 (Control), 14.81 ± 1.63 (3HTA), 4.37 ± 0.92 (3HTA + RR) and 2.41 ± 1.53 (3HTA + CsA/ADP); (C) 0.0018 ± 0.00015 (Control), 0.0646 ± 0.0176 (3HTA), 0.0044 ± 0.00021 (3HTA + RR) and 0.0008 ± 0.0004 (3HTA + CsA/ADP). Traces are representative of three independent experiments (animals) and are expressed as fluorescence arbitrary units (FAU) (A, B and D) or absorbance (C). ***$P < 0.001$ compared to controls (Duncan's multiple range test).

alter the tested parameters in brain and heart mitochondria. This may have occurred because dicarboxylic acids cannot enter into the mitochondrial matrix through the inner mitochondrial membrane by diffusion, as occurs with monocarboxylic fatty acids that are good protonophores [58,59]. Furthermore, it is conceivable that the LCHFAs 3HTA and 3HPA provoked selective deleterious effects on mitochondrial homeostasis because DCA, another long-chain fatty acid, had no effects on the tested parameters.

It is of interest that, under our experimental conditions, the capacity of heart mitochondria with respect to retaining Ca^{2+} and resisting mPT induction was higher than brain mitochondria after a sequence of Ca^{2+} pulses in the absence of LCHFAs (data not shown), highlighting the vulnerability of heart to the effects of LCHFAs.

It is difficult to establish the pathophysiological relevance of our present data because the concentrations of LCHFAs in the heart and brain of patients

affected by LCHAD and MTP deficiencies are still unknown. However, it is of interest that the heart, in contrast to the brain, mainly utilizes fatty acids for energy needs, and the enzymatic fatty acid oxidation steps, including those of the catabolism of LCHFAs, are highly expressed in this tissue [28,60]. Therefore, it is likely that concentrations of LCHFAs are probably higher in cardiac tissue in comparison to the brain. In this respect, it was demonstrated that concentrations of LCHFAs in cultured skin fibroblasts from LCHAD deficient patients exposed to palmitate can be 1 mM, or even higher, indicating that the intracellular concentration of these compounds may be extremely high in tissues with intense fatty acid catabolism. Accordingly, we found that the effects of LCHFAs were more pronounced and were obtained with lower concentrations in the heart (10–20 μM) compared to brain mitochondria (30–60 μM). These findings may possibly be associated with the characteristic clinical phenotype presented by patients
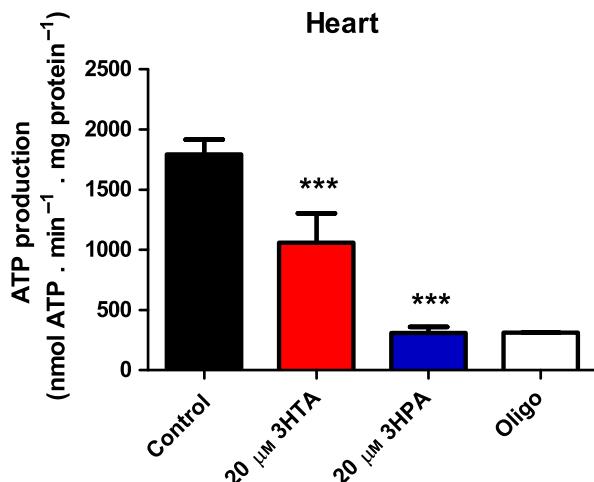
Heart

Fig. 6. Effects of 3HTA and 3HPA on ATP production in Ca^{2+}-loaded mitochondria from the heart. All experiments were performed in a reaction medium containing heart mitochondrial preparations (0.35 mg protein·mL^{-1}) supported by glutamate plus malate. 3HTA or 3HPA (20 μM) were added at the beginning of the assay. Controls were performed in the absence of fatty acids. Ninety seconds later, 30 μM Ca^{2+} was added. The medium was supplemented with ADP (1 mM) 90 s afterwards. Oligomycin A (Oligo) (1 μM·mL^{-1}) was used as a positive control. Values are the mean ± SD of six independent experiments (animals) (nmol ATP·min^{-1}·mg^{-1}). ***$P < 0.001$ compared to controls (Duncan's multiple range test).

affected by these diseases, with acute and sometimes lethal cardiomyopathy being associated with mild neurological symptoms. Furthermore, the higher oxidative capacity of the heart may be related to its higher susceptibility to 3HTA and 3HPA, reinforcing our presumption that heart physiology is severely compromised by mitochondrial dysfunction in LCHAD and MTP deficiencies. Finally, the robust disturbance of mitochondrial homeostasis caused by 3HPA and 3HTA was achieved with similar and even lower concentrations (10–30 μM) than those found in the plasma of the affected patients during crises of metabolic decompensation, as characterized by a dramatic increase of these levels, as well as acute heart alterations [61,62], implying that our results may potentially contribute to the cardiac damage of patients affected by LCHAD and MTP deficiencies.

Interestingly, patients affected by VLCAD deficiency also present a cardiac phenotype similar to that of LCHAD and MTP deficiencies, although the underlying mechanisms of the heart alterations have been attributed to energy deficiency [63]. It would be interesting to evaluate the effects provoked by the major fatty acids accumulating in this disease on mitochon-

drial functions to access whether lipotoxicity could be also considered as a contributing factor to cardiac damage in this disorder.

Conclusions

For the first time, we provide solid evidence indicating that LCHFAs accumulating most in LCHAD and MTP deficiencies disrupt mitochondrial energy and calcium homeostasis in the heart. We have demonstrated that bioenergetics failures were mainly secondary to mPT induction in Ca^{2+}-loaded mitochondria and much stronger in the heart than in the brain. Because the disruption of energy metabolism and the dysregulation of intracellular Ca^{2+} concentrations may be harmful to cellular functions, we propose that these pathomechanisms may contribute to the pathophysiology of LCHAD and MTP deficiencies, especially the severe cardiomyopathy presented by the affected patients.

Materials and methods

Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except for 3HTDA (96% purity), which was synthesized by Dr Ernesto Brunet (Universidad Autonoma de Madrid). Stock solutions of a racemic mixture (DL) of 3HTA, 3HPA, 3HTDA and DCA were prepared in EtOH to dissolve these fatty acids. Their final concentrations in the incubation medium were in the range 10–60 μM, whereas the EtOH concentration was always 1%. The chosen doses were previously used in other studies investigating the effects of the long-chain fatty acids on biochemical parameters and are similar to those found in plasma of the affected patients [23,40]. The same percentage of EtOH was present in controls and was found not to alter *per se* the parameters evaluated.

Animals

Thirty-day-old Wistar rats obtained from our breeding colony were used. The animals were maintained under a 12 : 12 h light/dark cycle in an air-conditioned colony room at constant temperature (22 ± 1 °C), with free access to water and 20% (w/w) protein commercial chow (Nuvital, Colombo, Brazil). The study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (eighth edition, 2011) and was approved by the Ethical Committee of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data.

Preparation of mitochondrial fractions

Mitochondrial preparations from heart in accordance with the method described by Ferranti *et al.* [64]. The heart was removed and immersed in a protease solution (0.0025 mg·mL^{-1}) in isolation buffer with EGTA and without BSA for 10 min. After washing in isolation buffer with EGTA and BSA, it was homogenized in a Potter glass homogenizer at 900 r.p.m. Then, the homogenate was centrifuged at 600 *g* for 5 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 9000 *g* for 8 min at 4 °C. The resultant pellet was resuspended in 5 mL of isolation buffer without EGTA and centrifuged at 9000 *g* for 10 min at 4 °C. The final pellet was resuspended in isolation buffer without EGTA at an approximate protein concentration of 12 mg·mL^{-1}.

Mitochondria were isolated from forebrain according to Rosenthal *et al.* [65], with slight modifications [66]. Animals were euthanized by decapitation, and the brain was removed and homogenized with a glass hand-held homogenizer in ice-cold mitochondria isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% BSA (free fatty acid) and 10 mM Hepes (pH 7.2). The homogenate was centrifuged at 2000 *g* for 3 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 12 000 *g* for 10 min at 4 °C. The pellet was resuspended in 5 mL of isolation buffer with EGTA in the presence of 20 μL of digitonin 10% and centrifuged again at 12 000 *g* for 10 min at 4 °C. The resultant pellet was resuspended in 5 mL of isolation buffer without EGTA and centrifuged at 12 000 *g* for 10 min at 4 °C. The final pellet was resuspended in isolation buffer without EGTA at an approximate protein concentration of 20 mg·mL^{-1}. Mitochondria obtained from the brain and heart were immediately used and assays were carried out in the absence or presence of Ca^{2+}.

The protein concentration in both preparations was measured by the method of Bradford [67] using BSA as standard.

Experimental procedures for the spectrofluorimetric and spectrophotometric assays

Measurements of ΔΨm, NAD(P)H content and Ca^{2+} retention capacity were performed using spectrofluorimetry, whereas heart mitochondrial swelling was measured by spectrophotometry. ATP formation was determined by luminescence. Mitochondrial incubations were carried out at 37 °C, with continuous magnetic stirring and the assays were conducted in the presence of 1 μg·mL^{-1} oligomycin A (resting respiration), except for ATP measurements, using mitochondria (0.5 mg protein·mL^{-1} for brain and 0.2 or 0.35 mg protein·mg·mL^{-1} for heart) supported by 2.5 mM glutamate plus 2.5 mM malate. 3HTA (10–60 μM), 3HPA (20–30 μM), CaCl$_2$ (30–40 μM), carbonyl cyanide

3-chlorophenyl hydrazine (CCCP) (3 μM) and alamethicin (Alm) (40 μg·mL^{-1}) were added as indicated where appropriate. 3HTDA (30 μM), a dicarboxylic acid analogue to HTA, was also tested in the assays. In some experiments, RR (1 μM), CsA (1 μM) and ADP (300 μM) were added to the incubation medium. Traces are representative of independent experiments carried out in mitochondrial preparations from the heart and brain of three animals and are expressed as arbitrary units, unless otherwise stated. Statistical analyses were also carried out by analyzing quantitatively the data obtained from the assays.

Mitochondrial membrane potential (ΔΨm)

The ΔΨm was estimated according to Akerman and Wikstrom [68] and Figueira *et al.* [69] in a medium containing 150 mM KCl, 5 mM MgCl$_2$, 0.1 mg·mL^{-1} BSA, 5 mM Hepes, 2 mM KH$_2$PO$_4$ and 30 μM EGTA (pH 7.2). The fluorescence of 5 μM cationic dye safranine O, a ΔΨm indicator, was followed at excitation and emission wavelengths of 495 and 586 nm. CCCP was added at the end of measurements to abolish ΔΨm.

Mitochondrial NAD(P)H

Mitochondrial matrix NAD(P)H autofluorescence was measured at 366 nm excitation and 450 nm emission wavelengths, in a medium containing 150 mM KCl, 5 mM MgCl$_2$, 0.1 mg·mL^{-1} BSA, 5 mM Hepes, 2 mM KH$_2$PO$_4$ and 30 μM EGTA (pH 7.2). CCCP was added at the end of the measurements to induce maximal NAD(P)H oxidation.

Mitochondrial swelling

Mitochondrial swelling from heart mitochondria was determined in 96-well plates as the decrease in the turbidity of the mitochondrial suspension measured at 540 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA), in a medium (final volume 250 μL) containing 150 mM KCl, 5 mM MgCl$_2$, 0.1 mg·mL^{-1} BSA, 5 mM Hepes, 2 mM KH$_2$PO$_4$ and 15 μM EGTA (pH 7.2). A decrease in the turbidity indicates an increase in mitochondrial swelling. Alm was added at the end of the experiment to provoke maximal swelling.

Brain mitochondrial swelling was determined by spectrofluorimetry at 540 nm using the same medium supplemented with 30 μM EGTA. Alm was added at the end of the experiments to provoke maximal swelling.

Mitochondrial Ca^{2+} retention capacity

Mitochondrial Ca^{2+} retention capacity was determined in a medium containing 150 mM KCl, 5 mM MgCl$_2$, 0.1 mg·mL^{-1} BSA, 5 mM Hepes, 2 mM KH$_2$PO$_4$ and 10 μM

EGTA (pH 7.2), following the external free Ca^{2+} levels using 0.2 μM Calcium Green-5N (Molecular Probes, Invitrogen, Carlsbad, CA, USA) at excitation and emission wavelengths of 506 and 532 nm, respectively [36]. A low concentration of ADP (30 μM) was present in the incubation medium containing brain mitochondrial preparations to achieve more consistent mitochondrial Ca^{2+} uptake responses [36]. At the end of the measurements, maximal Ca^{2+} release was induced by CCCP.

ATP production

Heart mitochondrial fractions (0.35 mg protein·mL^{-1}) were incubated in respiring medium containing 0.3 M sucrose, 5 mM Mops, 5 mM KH$_2$PO$_4$, 50 μM EGTA and 0.01% BSA (pH 7.4), using 2.5 mM malate plus 2.5 mM glutamate as substrates in a final volume of 500 μL. The reaction was started by the addition of 1 mM ADP and stopped after 2 min with 1 μg·mL^{-1} oligomycin A. Thereafter, the mitochondrial suspension was immediately treated with 10 μL of ice-cold 6 M HClO$_4$. After centrifugation at 21 000 *g* for 5 min at 4 °C, aliquots (400 μL) of the supernatant were neutralized with 100 μL of 1 M K$_2$HPO$_4$ and submitted to a new centrifugation at 21 000 *g* for 5 min at 4 °C. ATP was determined in the resulting supernatant by the firefly luciferin–luciferase assay system in accordance with the manufacturer's instructions [70,71]. The luminescence was measured in a SpectraMax M5 microplate spectrofluorimeter. In some experiments, oligomycin A was used as a control.

Statistical analysis

The results are presented as the mean ± SD unless otherwise stated. Assays were performed in triplicate and the mean was used for statistical analysis. Data were analyzed using one-way analysis of variance followed by the post-hoc Duncan's multiple comparison test when *F* was significant. $P < 0.05$ was considered statistically significant for differences between groups. All analyses were carried out using SPSS, version 19.0 (IBM Corp., Armonk, NY, USA).

Acknowledgements

Conflicts of interest

The authors declare that there are no conflicts of interest.

Author contributions

CC planned and performed the experiments, and analyzed the data. FHH and MDNR performed the experiments. AUA planned the experiments, analyzed the data and wrote the paper. MW provided financial support, planned the experiments, analyzed the data and wrote the paper.

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Capítulo II

Disturbance of Mitochondrial Functions Provoked by the Major Long-Chain Hydroxy Fatty Acids Accumulating in MTP and LCHAD Deficiencies in Skeletal Muscle

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I indicate Dr. Rodrigue Rossignol as the Editorial Board member for this
submission, since he is an expert in mitochondrial bioenergetics and
metabolic diseases.

Covering Letter

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January 05th, 2016

Dr. Geoffrey Laurent,
Editor-in-Chief,
International Journal of Biochemistry & Cell Biology,

Dear Dr. Laurent,

I am sending you our manuscript entitled **"Disturbance of Mitochondrial Functions Provoked by the Major Long-Chain Hydroxy Fatty Acids Accumulating in MTP and LCHAD Deficiencies in Skeletal Muscle"**, which we would like to submit for publication in International Journal of Biochemistry & Cell Biology.

This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (Eighth edition, 2011) and was approved by the Ethical Committee of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to use the minimal number of animals necessary to produce reliable scientific data.

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

This manuscript presents for the first time experimental evidence showing that the major long-chain monocarboxylic 3-hydroxy fatty acids (LCHFA) that accumulate in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and mitochondrial trifunctional protein (MTP) deficiencies provoke mitochondrial dysfunction in rat skeletal muscle by behaving as uncouplers, metabolic inhibitors and inductors of mitochondrial permeability transition, besides impairing the capacity of mitochondria to retain calcium. We therefore presume that the LCHFA-induced disturbance of mitochondrial functions may contribute to the skeletal muscle lesions presented by patients affected by LCHAD and MTP deficiencies.

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

Yours sincerely,

M. Wajner, MD, PhD

1 **Disturbance of Mitochondrial Functions Provoked by the Major Long-Chain**

2 **Hydroxy Fatty Acids Accumulating in MTP and LCHAD Deficiencies in Skeletal**

3 **Muscle**

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Abstract

The pathogenesis of the muscular symptoms and recurrent rhabdomyolysis that commonly affect patients with mitochondrial trifunctional protein (MTP) and long-chain 3-hydroxy-acyl-CoA dehydrogenase (LCHAD) deficiencies is still unknown. Therefore, we studied the effects of the major monocarboxylic 3-hydroxylated fatty acids (LCHFA) accumulating in these disorders, namely 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids, on important skeletal muscle mitochondrial functions. We observed that these LCHFA markedly increased resting (state 4) respiration, decreased ADP-stimulated (state 3) and the respiratory control ratio (RCR), apart from uncoupled respiration in skeletal muscle mitochondria. 3HPA provoked similar effects in permeabilized skeletal muscle fibers, which is an integrated cellular system. We also verified that 3HTA and 3HPA decreased mitochondrial membrane potential, NAD(P)H content and Ca^{2+} retention capacity in Ca^{2+}-loaded skeletal muscle mitochondria. 3HPA-induced decrease of mitochondrial membrane potential and Ca^{2+} retention capacity were totally prevented by the mitochondrial permeability transition (mPT) inhibitors cyclosporin A and ADP, as well as by ruthenium red, a mitochondrial Ca^{2+} uptake blocker, indicating mPT induction and the involvement of Ca^{2+} in these effects. Mitochondrial membrane fluidity was also increased by 3HPA. In contrast, the dicarboxylic analogue of 3HTA, 3HTDA, was not able to alter any of the tested parameters. Taken together, our data strongly indicate that 3HTA and 3HPA behave as metabolic inhibitors, uncouplers of oxidative phosphorylation and mPT inducers in skeletal muscle. It is proposed that these pathomechanisms disturbing mitochondrial homeostasis may explain at least in part the muscular symptoms and episodes of rhabdomyolysis characteristic of MTP and LCHAD deficiencies.

Abbreviations: ANT, adenine nucleotide translocator; ATC, atractilosyde; BSA, bovine serum albumin; CCCP, carbonyl cyanide 3-chlorophenyl hydrazine; CsA, cyclosporin A; EGTA, ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid; EtOH, ethanol; FAU, fluorescence arbitrary units; GDP, guanosine diphosphate; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]; 3HTDA, 3-hydroxytetradecanodioic acid; 3HTA, 3-hydroxytetradecanoic acid; 3HPA, 3-hydroxypalmitic acid; LCFA, long-chain fatty acid; LCHAD, long-chain 3-hydroxy-acyl-CoA dehydrogenase; LCHFA, long-chain 3-hydroxylated fatty acids; ΔΨm, mitochondrial membrane potential; mPT, mitochondrial permeability transition; MTP, mitochondrial trifunctional protein; RCR, respiratory control ratio; RR, ruthenium red.

3

1. Introduction

The mitochondrial trifunctional protein (MTP) is an enzyme complex coupled to the inner mitochondrial membrane that catalyzes the oxidation of long-chain 3-hydroxy fatty acids (LCHFA). It is composed of three enzyme activities: long-chain enoyl-CoA hydratase, long-chain ketoacyl-CoA thiolase and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD). Molecular defects in MTP-encoding genes lead to deficiencies of isolated enzymatic activities, being the more common LCHAD deficiency, or of all three enzymatic components characterizing MTP deficiency (Rinaldo et al., 2002, Sander et al., 2005, Das et al., 2006).

MTP and LCHAD deficiencies are biochemically characterized by accumulation of long-chain 3-hydroxylated fatty acids (LCHFA) in body fluids and tissues, as well as by episodes of lactic acidemia and hypoketotic hypoglycemia, particularly during periods of intense catabolism, such as prolonged fasting and infections (Costa et al., 1998, Hintz et al., 2002, Olpin et al., 2005, Sander et al., 2005, Jones et al., 2001). Affected patients present a wide variety of symptoms, including severe hepatopathy and cardiomyopathy, as well as retinopathy, hypotonia, peripheral neuropathy, speech and developmental delay, lethargy and seizures (Wilcken, 2010, Waisbren et al., 2013). Skeletal myopathy associated with rhabdomyolysis, as well as general muscle weakness and pain, exercise intolerance and increased creatine kinase levels are frequent findings in patients with LCHAD and MTP deficiencies (den Boer et al., 2002, Diekman et al., 2014, Spiekerkoetter et al., 2004, Tyni and Pihko, 1999).

Acute cardiac manifestations with life-threatening arrhythmias and liver dysfunction may also occur in a considerable number of patients affected by MTP and LCHAD deficiencies during metabolic crises, whereas the late-onset forms are clinically milder and

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mainly characterized by neuromyopathic signs (Spiekerkoetter et al., 2004, Spiekerkoetter et al., 2008, Moczulski et al., 2009, den Boer et al., 2003, Tyni et al., 1997, Rocchiccioli et al., 1990).

Treatment is based on dietary fat restriction, fasting avoidance, night feeds, as well as supplementation of essential fatty acids and medium chain triglycerides (Gillingham et al., 1999, Saudubray et al., 1999, Spiekerkoetter and Wood, 2010, Haglind et al., 2013).

The pathophysiology of tissue damage in LCHAD and MTP deficiencies is still unclear, although hypoglycemia and hypoketonemia leading to energy deprivation may play a role. Considering the long-term high energy demand of muscular tissues is high and mainly obtained from fatty acid oxidation, it is possible that skeletal muscle damage and the occurrence of rhabdomyolysis in LCHAD and MTP deficiencies may involve low energy production due to a blockage of fatty acid oxidation. Alternatively, it may be presumed that the accumulating LCHFA and/or derivatives in these disorders are toxic to tissues, including the skeletal muscle (Wajner and Amaral, 2015). Furthermore, hyperlactic acidemia associated with abnormal mitochondrial morphology with swollen appearance and fat infiltration observed in the skeletal muscle of these patients indicate an important role for mitochondrial energy disruption in their pathogenesis (Tyni et al., 1996, Ventura et al., 1998). This presumption is supported by previous studies demonstrating that the monocarboxylic LCHFA accumulating in LCHAD and MTP deficiencies disturb mitochondrial homeostasis in forebrain, liver and heart of young rats (Tonin et al., 2010, Tonin et al., 2013, Tonin et al., 2014), but, to the best of our knowledge, nothing has been reported regarding the toxic effects of the compounds on skeletal muscle mitochondrial functions.

119   Thus, in the present work we focused our attention on the role of the 3-

120   hydroxytetradecanodioic (3HTDA), 3-hydroxytetradecanoic (3HTA) and 3-

121   hydroxypalmitic (3HPA) acids that mostly accumulate in LCHAD and MTP deficiencies

122   on important parameters of mitochondrial bioenergetics, namely the respiratory states 3 and

123   4, respiratory control ratio (RCR) and uncoupled respiration, as well as the mitochondrial

124   membrane potential ($\Delta\Psi$m), Ca^{2+} retention capacity and matrix NAD(P)H content in

125   skeletal muscle mitochondrial preparations from young rats in the presence or absence of

126   Ca^{2+} in order to contribute to the understanding of the pathomechanisms of muscle lesions

127   that usually affect LCHAD and MTP deficient patient.

128

129   **2. Material and methods**

130

131   **2.1. Reagents**

132   All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except

133   for 3HTDA (96% purity), which was synthesized by Dr. Ernesto Brunet, from Universidad

134   Autonoma de Madrid, and Laurdan that was purchase from Invitrogen (Molecular Probes).

135   Stock solutions of a racemic mixture (DL) of 3HTDA, 3HTA and 3HPA were prepared in

136   ethanol (EtOH) to dissolve the fatty acids and added to incubation medium at final

137   concentrations of 10 – 100 μM. The final concentration of EtOH in the incubation medium

138   was 0.3 %. For the experiments performed with muscle fibers, 3HPA was dissolved in

139   dimethyl sulfoxide (DMSO) in a final concentration of 0.3 % in the medium. The same

140   percentage of EtOH or DMSO was present in controls and proved not to alter *per se* the

141   parameters evaluated.

2.2. Animals

143   Thirty-day-old Wistar rats obtained from our breeding colony were used. The

144 animals were maintained on a 12:12 h light/dark cycle in air-conditioned constant

145 temperature (22 ± 1°C) colony room, with free access to water and 20% (w/w) protein

146 commercial chow. This study was performed in strict accordance with the Guide for the

147 Care and Use of Laboratory Animals (Eighth edition, 2011) and approved by the Ethical

148 Committee of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts

149 were also made to use the minimal number of animals necessary to produce reliable

150 scientific data.

151

152 **2.3. Preparation of mitochondrial fractions**

153   Mitochondrial preparations from skeletal muscle were isolated according to Chweih

154 2015, with modifications. The animals were sacrificed by decapitation and four quadriceps

155 samples (2 animals) were trimmed from connective tissue and finely diced with scissors in

156 ~10 ml of isolation medium I (100 mM sucrose, 50 mM KCl, 5 mM EDTA,

157 1 mM K_2HPO_4 and 2 mg . mL^{-1} BSA; 50 mM Tris, pH 7.4). Then, the samples were

158 subjected to two 3 seconds bouts of homogenization in a Tissue Homogenizer Omni

159 (Kennesaw, GA, USA) bladed homogenizer and after homogenized in a Potter glass

160 homogenizer (15 mL) at 900 r.p.m. The homogenate was then and centrifuged at 800g for

161 10 min. This supernatant was subsequently centrifuged at 10,000g for 10 min, and the

162 resulting pellet containing mitochondria was suspended gently in isolation medium I with

163 the aid of a tiny paintbrush. The suspension was centrifuged again at 10,000g for 10 min,

164 and the pellet was rinsed twice with fresh changes of isolation medium II

165 (225 mM mannitol, 75 mM sucrose and 10 mM Tris, pH 7.4) prior to its suspension in the

166 same medium at a protein concentration of 15 mg. mL^{-1}. All the isolation procedures were

167 conducted on ice or at 4°C.

168   Protein concentration was measured by the method of Bradford (1976) (Bradford,

169 1976), using BSA as standard. Mitochondria obtained from skeletal muscle were used in

170 the assays immediately after isolation and assays were carried out in the absence or

171 presence of Ca^{2+}.

172

173 **2.4. Preparation of skeletal muscle fibers**

174   Plantar muscle was harvested from rats and placed in ice-cold relaxing solution

175 containing 10 mM Ca-EGTA buffer (2.77 mM of CaK_2EGTA + 7.23 mM K_2EGTA), free

176 calcium concentration of 0.1 mM, 20 mM imidazole, 50 mM K^+/4-

177 morpholinoethanesulfonicacid, 0.5 mM dithiothreitol, 7 mM $MgCl_2$, 5 mM ATP, 15 mM

178 phosphocreatine, pH 7.1. Approximately 8 milligrams of plantar skeletal muscle were

179 utilized and individual fiber bundles were separated with 2 forceps. Samples were

180 permeabilized for 30min in ice-cold relaxing solution with saponin (50μg/mL) gently

181 stirred and washed 3 times with MiR05 medium (60 mM potassium lactobionate, 0.5 mM

182 EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose,

183 1 mg . mL^{-1} BSA, pH 7.1) at 4∘C. Samples were dried with filter paper and weighted

184 (Kuznetsov et al., 2008).

185

186 **2.5. Respiratory parameters determined through mitochondrial oxygen consumption**

187   The rate of oxygen consumption was measured using an OROBOROS Oxygraph-2k

188 (Innsbruck, Austria) in a thermostatically controlled (37°C) and magnetically stirred

189 incubation chamber. 3HTA or 3HPA (10–100 μM) were added to the reaction medium

190 after 60 seconds of the beginning of the assay. The assay was performed with

191 mitochondrial preparations (0.25 mg protein . mL^{-1} using 2.5 mM glutamate plus 2.5 mM

192 malate as substrate) incubated in a buffer containing 150 mM KCl, 1 mM MgCl$_2$, 0.1 mg .

193 mL^{-1} BSA, 2 mM KH$_2$PO$_4$, 200 μM EGTA, 5 mM HEPES, pH 7.2. State 3 respiration was

194 measured after addition of 1 mM ADP to the incubation medium and uncoupled respiration

195 after addition of 1 μM CCCP. In order to measure resting (state 4) respiration, 1 μg. mL^{-1}

196 oligomycin A was added to the incubation medium. In some experiments, guanosine

197 diphosphate (GDP, 200 μM) and atractilosyde (ATC, 30 μM) were added to the

198 mitochondrial preparations. The RCR (state 3/state 4) was then calculated. States 3 and 4

199 were expressed as ρmol O$_2$ consumed. s^{-1}. mg protein^{-1}. Only mitochondrial preparations

200 with RCR greater than 5 were used in the experiments.

201   We also measured oxygen consumption in the permeabilized skeletal muscle fibers

202 in a medium MiR05 at 37ºC, in the presence of 10 mM glutamate and 5 mM malate using

203 OROBOROS (Innsbruck, Austria). 3HPA (50 and 100 μM) was pre-incubated at 37 ºC for

204 15 minutes, before addition of substrates. Ten minutes afterwards, ADP (400 μM) was

205 added to the incubation medium (state 3 respiration). State 4 respiration was induced by 1

206 μg/mL oligomycin 15 minutes after ADP addition. Finally, 1 μM CCCP was added to

207 stimulate the uncoupled respiration in the muscle fibers (Kuznetsov et al., 2008).

208

209 **2.6. Experimental procedures for the spectrofluorimetric assays**

210   Measurements of ΔΨm, NAD(P)H content and Ca^{2+} retention capacity were

211 performed using spectrofluorimetry. Mitochondrial incubations were carried out at 37°C,

212 with continuous magnetic stirring and the assays were conducted in the presence of 1 μg .

213 mL^{-1} oligomycin A (resting respiration) using skeletal muscle mitochondria (0.5 mg protein

214 . mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate. 3HTDA (30 μM), 3HTA (30

215 μM), 3HPA (10-30 μM), CaCl$_2$ (30 μM) and CCCP (3 μM) were added as indicated by the

216 arrows in the figures. In some experiments, ruthenium red (RR, 1 μM), cyclosporin A

217 (CsA, 1 μM) and ADP (300 μM) were added in the assay. Traces are representative of

218 independent experiments carried out in mitochondrial preparations from skeletal muscle of

219 three animals and were expressed as arbitrary units, unless otherwise stated. Statistical

220 analyses were also carried out by analyzing quantitatively the data obtained from the

221 assays.

222

223 **2.7. Mitochondrial membrane potential (ΔΨm)**

224 The ΔΨm was estimated according to Akerman and Wikstrom (1976) (Akerman

225 and Wikstrom, 1976) and Figueira and collaborators (2012) (Figueira et al., 2012) in

226 medium containing 150 mM KCl, 5 mM MgCl$_2$, 0.1 mg . mL^{-1} BSA, 2 mM KH$_2$PO$_4$, 30

227 μM EGTA, 5 mM HEPES, pH 7.2. The fluorescence of 5 μM cationic dye safranine O, a

228 ΔΨm indicator, was followed at excitation and emission wavelengths of 495 and 586 nm.

229 CCCP was added in the end of measurements to abolish ΔΨm.

230

231 **2.8. Mitochondrial NAD(P)H**

232 Mitochondrial matrix NAD(P)H fluorescence was measured at 340 nm excitation

233 and 450 nm emission wavelengths, in medium containing 150 mM KCl, 5 mM MgCl$_2$, 0.1

234 mg . mL^{-1} BSA, 2 mM KH$_2$PO$_4$, 30 μM EGTA, 5 mM HEPES, pH 7.2. CCCP was added

235 in the end of the measurements to induce maximal NAD(P)H oxidation.

2.9. Mitochondrial Ca^{2+} retention capacity

Ca^{2+} retention capacity was determined in medium containing 150 mM KCl, 5 mM MgCl$_2$, 0.1 mg . mL^{-1} BSA, 2 mM KH$_2$PO$_4$, 10 μM EGTA, 5 mM HEPES, pH 7.2, following the external free Ca^{2+} levels using 0.2 μM Calcium Green-5N (Molecular Probes, Invitrogen, Carlsbad, CA) at excitation and emission wavelengths of 506 and 532 nm, respectively (Saito and Castilho, 2010). In the end of the measurements, maximal Ca^{2+} release was induced by CCCP.

2.10. Mitochondrial membrane fluidity

Mitochondrial membrane fluidity was determined by measuring the fluorescence intensity of laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) in 96-well plates. Freshly prepared skeletal muscle mitochondria were suspended (0.5 mg . ml^{-1}) in a medium containing 150 mM KCl, 1 mM MgCl$_2$, 0.1 mg . mL^{-1} BSA, 2 mM KH$_2$PO$_4$, 200 μM EGTA, 5 mM HEPES, pH 7.2, 2.5 mM glutamate plus 2.5 mM malate and 1 μg . mL^{-1} oligomycin A, and incubated with 50 μM 3HPA for 5 minutes. After that, 1 μM laurdan was added and the samples and incubated for 30 min at 37 °C protected from light. Laurdan fluorescence was followed at excitation wavelengths of 360 nm and emission of 440 and 490 nm using a SpectraMax M5 microplate reader. The generalized polarization (GP) readings were calculated as follows:

GP = (I$_{440}$ − I$_{490}$) / (I$_{440}$ + I$_{490}$), where I$_{440}$ and I$_{490}$ were the laurdan emission intensities at the respective wavelengths (Zaccagnino et al., 2012, Luevano-Martinez et al., 2015).

2.11. Statistical analysis

260   Results are presented as mean ± standard deviation, unless otherwise stated. Assays

261 were performed in triplicate and the mean was used for statistical calculations. Data were

262 analysed by one-way ANOVA, followed by the post-hoc Duncan multiple range test when

263 multiple comparisons were performed. Student´s *t* test for paired samples was used when a

264 single parameter was compared between two different groups. Differences between groups

265 were rated significant at P < 0.05. Analyses of ANOVA were carried out using the 19.0

266 SPSS Statistics software and Student's t test by GraphPad Prism.

267

268 **3. Results**

269

270 **3.1. Long-chain monocarboxylic 3-hydroxylated fatty acids alter oxygen consumption**

271 **in skeletal muscle mitochondria**

272   We initially investigated the effects of 3HTA and 3HPA (10 – 100 μM) on oxygen

273 consumption using glutamate plus malate-supported in skeletal muscle mitochondria in a

274 medium devoid of Ca^{2+} (containing 200 μM EGTA). 3HTA and 3HPA strongly increased

275 resting mitochondrial respiration (state 4) in skeletal muscle mitochondria at concentrations

276 as low as 10 μM, indicating an uncoupling behavior, whereas higher levels of these fatty

277 acids (100 μM 3HTA and 50-100 μM 3HPA) provoked an opposite effect inhibiting state 4

278 respiration (Fig 1A: [$F_{(8,18)}$=41.959, P<0.001]). Furthermore, skeletal muscle mitochondria

279 in a medium devoid of Ca^{2+} (containing 200 μM EGTA). 3HTA and 3HPA also decreased

280 ADP-stimulated (state 3) (Fig 1B: [$F_{(8,18)}$=64.132, P<0.001]) and uncoupled respiration (Fig

281 1C: [$F_{(8,18)}$=28.165, P<0.001]) in a dose dependent manner, that is characteristic of a

282 metabolic inhibition. It can be also seen in the figure that 3HPA provoked stronger effects

283 when compared to 3HTA. It is stressed that the inhibitory effects of high concentrations of

284 these fatty acids on state 4 respiration may be due to their role as metabolic inhibitors. In

285 addition, RCR was markedly decreased by 3HTA and 3HPA (Fig 1D: [$F_{(8,18)}$=24.702,

286 P<0.001]), reinforcing their role as uncouplers of mitochondrial oxidative phosphorylation.

287 We also observed that 3HPA-induced increase of state 4 respiration in skeletal muscle

288 mitochondria was not affected by the competitive adenine nucleotide translocator (ANT)

289 inhibitor ATC and by the UCP inhibitor GDP (Fig 2: [$F_{(5,12)}$=143.227, P<0.001]), making

290 unlikely the involvement of ANT and UCP in 3HPA toxicity and indicating other

291 mechanisms for this effect.

292

293 **3.2. 3-Hydroxypalmitic acid (3HPA) decreases ADP- and CCCP-stimulated**

294 **respiration in skeletal muscle fibers**

295   In order to analyze the effects provoked by LCHFA on mitochondrial functions *in*

296 *situ*, we tested the influence of 3HPA (50-100 uM) on mitochondrial respiration using

297 permeabilized skeletal muscle fibers. We found that 3HPA strongly decreased oxygen

298 consumption stimulated by ADP (Fig 3: [$F_{(2,6)}$=81.149, P<0.001) or CCCP (Fig 3:

299 [$F_{(2,6)}$=119.287, P<0.001]), indicating that 3HPA behaves as a metabolic inhibitor in an

300 integrated cellular system, corroborating our data obtained using isolated mitochondria.

301

302 **3.3. Long-chain monocarboxylic 3-hydroxylated fatty acids reduce ΔΨm in skeletal**

303 **muscle mitochondria**

304   We then assessed the influence of 3HTDA, 3HTA and 3HPA on ΔΨm in skeletal

305 muscle mitochondria supported by glutamate plus malate. It was observed that low

306   concentrations of 3HTA and 3HPA (10-30 µM) provoked a strong dissipation of $\Delta\Psi$m

307   when mitochondria were challenged by Ca^{2+} in a medium with low EGTA concentration

308   (Fig 4A: [$F_{(3,8)}$=264.306, P<0.001]; 4B: [$F_{(3,8)}$=12.061, P<0.01]). Furthermore, RR, an

309   inhibitor of mitochondrial Ca^{2+} uptake, totally abolished the reduction of $\Delta\Psi$m caused by

310   3HPA, implying the importance of Ca^{2+} in this effect. Similarly, the classical inhibitors of

311   mitochondrial permeability transition (mPT), CsA and ADP, also protected glutamate plus

312   malate-supported mitochondria (Fig 4C: [$F_{(2,6)}$=11.106, P<0.01]) against membrane

313   depolarization, supporting therefore an induction of mPT pore opening. In contrast, the

314   dicarboxylic 3HTDA did not alter $\Delta\Psi$m under the same experimental conditions (Fig 5A).

315

316   **3.4. Long-chain monocarboxylic 3-hydroxylated fatty acids decrease NAD(P)H matrix**

317   **mitochondrial content in skeletal muscle mitochondria**

318   In the next set of experiments we evaluated the effects of 3HTDA, 3HTA and

319   3HPA on mitochondrial NAD(P)H content in skeletal muscle mitochondria supported by

320   glutamate plus malate. We observed that 3HTA and 3HPA (30 µM) provoked a significant

321   decrease of NAD(P)H content when Ca^{2+} was added to the medium that contained a low

322   concentration of EGTA (Fig 5: [$F_{(3,8)}$=10.467, P<0.01]), suggesting that the reduced

323   equivalents were oxidized or lost from the matrix probably due to mPT. Furthermore, it can

324   be seen in the figure that 3HTDA, the dicarboxylic analogue of 3HTA, did not change

325   NAD(P)H content both in the presence or absence of Ca^{2+}.

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3.5. Long-chain monocarboxylic 3-hydroxylated fatty acids impair Ca²⁺ retention capacity in skeletal muscle mitochondria

Since mPT induction may compromise mitochondrial Ca^{2+} homeostasis and lead to release of this cation from the mitochondria, we determined the mitochondrial Ca^{2+} retention capacity in the presence of 3HTDA, 3HTA or 3HPA using glutamate plus malate or succinate as substrates. It can be seen in Fig 6 that 3HTA and 3HPA (10-30 µM) significantly reduced the mitochondrial Ca^{2+} retention capacity (panels A and B) and that this effect that was completely prevented by CsA plus ADP (panel C). In contrast, 3HTDA did not alter mitochondrial Ca^{2+} retention capacity (panel A).

3.6. 3-Hydroxypalmitic acid (3HPA) increases mitochondrial membrane fluidity

We also determined whether 3HPA might alter skeletal muscle mitochondrial membrane fluidity by following GP of laurdan fluorescence, since alterations of membrane fluidity may compromise mitochondrial functions (Eckmann et al., 2013) and be a trigger factor for mPT induction (Colell et al., 2003). It was found that 3HPA markedly reduced GP of laurdan fluorescence (Fig 7: t=3.608, P<0,05). Since membrane fluidity is inversely proportional to the ratio used for GP calculation (Palleschi and Silvestroni, 1996), our data indicate that this fatty acid increase the mitochondrial membrane fluidity.

4. Discussion

Patients affected by MTP and LCHAD deficiencies usually present myopathy, characterized by muscular weakness and pain. Conditions leading to an increased energy demand, such as fasting, exercise, infections or febrile illness may induce episodes of rhabdomyolysis and increased creatine kinase levels (Houten and Wanders, 2010,

353   Spiekerkoetter, 2010, Olpin et al., 2005, Diekman et al., 2014). The underlying mechanisms

354   responsible for these symptoms muscular weakness and pain, exercise intolerance and

355   increased creatine kinase levels are still poorly known, although lactic acidosis that occurs

356   in some affected patients indicate mitochondrial dysfunction.

357   In the present work we found that 3HTA and 3HPA, the major LCHFA

358   accumulating in MTP and LCHAD deficiencies, disrupt important mitochondrial functions

359   (energy and calcium homeostasis) in skeletal muscle of young rats at concentrations as low

360   as 10 μM. 3HPA and 3HTA compromised the maintenance of $\Delta\Psi$m and the matrix

361   NAD(P)H pool, as well as Ca^{2+} retention capacity, by uncoupling oxidative

362   phosphorylation, inducing MTP and behaving as metabolic inhibitors in skeletal muscle

363   mitochondria.

364   It was first verified that 3HPA and 3HTA increased state 4 respiration and

365   decreased RCR that is characteristically observed in uncouplers of oxidative

366   phosphorylation. Interestingly, at higher concentrations (100 μM 3HTA and 50-100 μM

367   3HPA) these compounds decreased state 4 respiration, indicating that they may also act as

368   metabolic inhibitors. This presumption was supported by the significant decrease of state 3

369   and uncoupled mitochondrial respiration caused by these LCHFA in a dose dependent

370   manner, with 3HPA showing more marked effects as compared to 3HTA.

371   The uncoupling effect induced by 3HPA in skeletal muscle mitochondria could not

372   be attributed to ANT and UCP as occurs for non-hydroxylated long chain fatty acids

373   (LCFA), since the ANT inhibitor ATC and the UCP inhibitor GDP did not change 3HPA-

374   induced increase of resting respiration (state 4). Therefore, our data indicate that the

375   hydroxylated LCFA uncouple oxidative phosphorylation in skeletal muscle mitochondria

376   by a different mechanism than those of the non-hydroxylated LCFA. Thus, it is feasible that

377   the uncoupling of oxidative phosphorylation could be due to a protonophoric mechanism

378   due to the transbilayer movement of undissociated (linked to protons) LCHFA through the

379   mitochondrial inner membrane towards the mitochondrial matrix. The passage of

380   dissociated fatty acids in the opposite direction to the intermembrane mitochondrial space is

381   usually facilitated by ANT and UCP (Schonfeld et al., 1989, Schonfeld, 1992, Goglia and

382   Skulachev, 2003, Affourtit et al., 2007). However ANT and UCP carriers seem not be

383   involved in the uncoupling effects here observed for the LCHFA, so that we cannot discard

384   that LCHFA anions may also be transferred by other mitochondrial carriers, such as the

385   glutamate/aspartate antiporter (Skulachev, 1998, Mokhova and Khailova, 2005), the mono-

386   and tri-carboxylate carriers (Schonfeld et al., 2000) and the phosphate carrier (Zackova et

387   al., 2000). LCHFA-induced uncoupling of oxidative phosphorylation may alternatively

388   have occurred at least in part due to increased mitochondrial membrane fluidity provoked

389   by these fatty acids, as here demonstrated for 3HPA in skeletal muscle mitochondria (Zhao

390   and Hirst, 1990, Schroder et al., 1996).

391   On the other hand, our observations showing that 3HPA also behaves as a strong

392   metabolic inhibitor in permeabilized skeletal muscle fibers make our data obtained in

393   isolated mitochondria of pathophysiological relevance. It is stressed that this *in situ*

394   approach allows the analysis of mitochondria within an integrated cellular system in their

395   normal intracellular position and assembly, preserving essential interactions with the

396   cytoskeleton, nucleus and endoplasmic reticulum (Kuznetsov et al., 2008).

397   3HTA and 3HPA also strongly dissipated $\Delta\Psi m$ at 10-30 μM in the presence of Ca^{2+}

398   and mitochondrial depolarization was fully prevented by RR, a known inhibitor of the

399   mitochondrial Ca^{2+} uniporter (Moore, 1971), and also by CsA plus ADP, classical inhibitors

400   of MPT (Rottenberg and Marbach, 1989, Saito and Castilho, 2010). In this context, CsA is

17

401 an inhibitor of MPT by inactivating the cyclophilin D, a mitochondrial matrix protein

402 associated with the mPT occurrence (Tanveer et al., 1996, Baines et al., 2005, Basso et al.,

403 2005, Bernardi, 2013), whereas ADP is a potent inhibitor of mPT in isolated mitochondria

404 by binding to ANT in the matrix side. Taken together, these data indicate an induction of

405 mPT provoked by low concentrations of these fatty acids.

406   Another novel finding of our investigation was the observation that the major

407 hydroxylated fatty acids accumulated in LCHAD and MTP deficiencies strongly impaired

408 the mitochondrial capacity to uptake and retain Ca^{2+} in skeletal muscle, that is another

409 important function of mitochondria (Baughman et al., 2011, De Stefani et al., 2011, Pan et

410 al., 2013, Marchi and Pinton, 2014, Pendin et al., 2014). This effect was totally prevented

411 by CsA and ADP, implying that mPT induction is associated with the reduction of

412 mitochondrial Ca^{2+} retention capacity (Zoratti and Szabo, 1995, Crompton et al., 1999,

413 Bernardi and von Stockum, 2012). In this scenario, it is emphasized that mitochondria play

414 an essential role in cellular Ca^{2+} homeostasis, as well as in the excitation-contraction

415 mechanism of muscle fibers by rapidly capturing Ca^{2+} to shape the cytosolic Ca^{2+} transients

416 in skeletal muscle during excitation-contraction coupling (Yi et al., 2011). Noteworthy, the

417 capacity of mitochondria from skeletal and cardiac muscle to retain Ca^{2+} is much more

418 pronounced as compared to brain and liver mitochondria under our experimental conditions

419 (Hickmann et al., 2015, Cecatto et al., 2015, Tonin et al., 2014).

420   On the other hand, considering the important role of mitochondria in buffering

421 cytosolic Ca^{2+} in myocytes (Yi et al., 2011) and since imbalance of intracellular calcium

422 concentration has been also suggested as a pathophysiologic event leading to

423 rhabdomyolysis (Hamel et al., 2015), it is possible that induction of mPT leading to

424 mitochondrial Ca^{2+} imbalance may also represent a potential mechanism involved in these

425 mitochondrial morphological alterations. This presumption is in accordance with a recent

426 work demonstrating that NIM-811 treatment, a derivative of CsA, prevented and reduced

427 rhabdomyolysis of the skeletal muscle in an experimental model of lower limb arterial

428 occlusive diseases and isquemic-reperfusion injuries during vascular operations (Garbaisz

429 et al., 2014).

430 The impairment of mitochondrial functions by the major accumulating LCHFA may

431 possibly be related to the increase of lactic acid concentrations and the mitochondrial

432 morphological abnormalities observed in skeletal muscle from patients affected by

433 MTP/LCHAD deficiencies (Amirkhan et al., 1997, Tyni et al., 1996, Ventura et al., 1998,

434 Das et al., 2000, Enns et al., 2000). Furthermore, considering that the skeletal muscle is

435 highly dependent of their mitochondrial oxidative system, we may presume that the

436 episodes of rhabdomyolysis associated with muscular lesions may also be related to the

437 LCHFA-induced decrease of the oxidative capacity in this tissue, especially during

438 metabolic crises characterized by accelerated catabolism and lipolysis provoking

439 substantial increase of the accumulating fatty acids.

440 The nonselective permeabilization caused by mPT activation may lead to

441 mitochondrial swelling and loss of elements (Ca^{2+}, Mg^{2+}, glutathione, NADH and NADPH)

442 from the matrix, including pro-apoptotic factors such as cytochrome c, potentially leading

443 to apoptosis or/and necrosis. mPT activation also impairs oxidative phosphorylation and

444 ATP synthesis (Zoratti and Szabo, 1995, Starkov, 2010, Rasola and Bernardi, 2011,

445 Bernardi and von Stockum, 2012, Rizzuto et al., 2012, Crompton et al., 1999, Figueira et

446 al., 2013). Indeed, we found that 3HTA and 3HPA (30 μM) also provoked a decrease of

447 mitochondrial matrix NAD(P)H content after Ca^{2+} loading, which may be secondary to a

448 nonselective inner membrane permeabilization due to mPT. Alternatively, NADH

19

449   oxidation or consumption due to mPT induction and consequent activation of the electron

450   transport flow may have also contributed to the reduction of NAD(P)H concentrations as

451   here detected (Le-Quoc and Le-Quoc, 1989, Kehrer and Lund, 1994, Kowaltowski et al.,

452   2001).

453   As regards to the mechanisms involved in the nonselective permeabilization

454   induced by mPT pore opening, we point out uncoupling of oxidative phosphorylation

455   (Bernardi, 1992, Castilho et al., 1997, Kowaltowski et al., 1996, Zago et al., 2000),

456   oxidative attack to the membrane protein thiol groups on the mPT pore [48-49] and

457   interaction with mitochondrial membrane phospholipids such as cardiolipin (Paradies et al.,

458   2014). Since our data clearly demonstrated that 3HTA and 3HPA acted as potent

459   uncouplers, it is presumed that uncoupling of oxidative phosphorylation may underlie mPT

460   induction provoked by these fatty acids.

461   Alterations of mitochondrial membrane fluidity leading to conformational changes

462   during the assembly of membrane proteins that constitute the pore may also potentially

463   represent an underlying mechanism responsible for LCHFA-induced mPT pore opening

464   (Ricchelli et al., 1999). This was probably the case since we have shown that 3HPA

465   significantly increased membrane fluidity in skeletal muscle mitochondria. Interestingly,

466   reduced mitochondrial membrane fluidity seems to make this organelle more resistant to

467   undergo mPT (Colell et al., 2003).

468   We also found that the dicarboxylic acid LCHFA 3HTDA, a structurally analogue

469   of the monocarboxylic 3HTA, did not change the tested parameters, implying a selective

470   action of the monocarboxylic acids 3HTA and 3HPA. In this respect, it is possible that the

471   lack of effects of the dicarboxylates could be because of their inability to cross the inner

472   mitochondrial membrane by diffusion as occurs with the monocarboxylic analogues (Jezek

473 et al., 1997, Skulachev, 1991, Liu et al., 1996) or because monocarboxylic LCFA are in

474 general good protonophores (uncouplers), as compared to the dicarboxylate analogues

475 (Wojtczak et al., 1998).

476   The data shown in the present work are in accordance with previous findings

477 showing that monocarboxylic LCHFA disrupt mitochondrial homeostasis in heart, liver and

478 brain (Cecatto et al., 2015, Hickmann et al., 2015, Tonin et al., 2014). These studies

479 associated with the present findings obtained in skeletal muscle strongly indicate that the

480 great variety of clinical symptoms presented by MTP and LCHAD deficient patients,

481 including skeletal muscle weakness, rhabdomyolysis, cardiomyopathy, hepatopathy and

482 encephalopathy may be tentatively related to mitochondrial lipotoxicity induced by the

483 major LCHFA accumulated in these disorders. It is of note that skeletal and cardiac muscle

484 mitochondria were much more susceptible to the toxic effects provoked by 3HTA and

485 3HPA, followed by liver and brain mitochondria, implying an important role of LCHFA in

486 the pathogenesis of skeletal muscle lesions characteristic of MTP and LCHAD deficient

487 patients.

488   It is difficult to establish the pathophysiological relevance of our data since the

489 muscle concentrations of free LCHFA in patients affected by LCHAD and MTP

490 deficiencies are still unknown. However, since fatty acids are the main substrate for energy

491 production in skeletal muscle, it is conceivable that a blockage of this pathway as occurs in

492 MTP and LCHAD deficiencies leads to accumulation of these fatty acids deregulating

493 mitochondrial functions and therefore contributing to the pathophysiology of these

494 diseases. It is also emphasized that 3HPA and 3HTA, the major accumulating metabolites,

495 provoked significant effects at similar or even lower concentrations (10 μM) than those

496 found in plasma of the affected patients, especially during crises of metabolic

decompensation that generally follow infections and accelerated catabolism that are associated with increased tissue levels of these fatty acids and with worsening of the clinical symptoms (Hagenfeldt et al., 1990, Costa et al., 1998).

5. Conclusions

In conclusion, we provide for the first time experimental evidence that the monocarboxylic LCHFA that most accumulate in LCHAD and MTP deficiencies behave as potent metabolic inhibitors, uncoupling agents and mPT inducers in skeletal muscle from young rats. Since these effects compromise mitochondrial energy and Ca^{2+} homeostasis, we propose that disturbance of mitochondrial functions may be associated with lactic academia, muscle damage and recurrent episodes of rhabdomyolysis presented by patients affected by LCHAD and MTP deficiencies.

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773

774 **Legends to figures**

775

776 **Fig 1. Effects of 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids**

777 **on oxygen consumption in skeletal muscle mitochondria.** (A) Resting (state 4), (B)

778 ADP-stimulated (state 3), (C) uncoupled (CCCP-stimulated) respiration and (D) respiratory

779 control ratio (RCR). Glutamate plus malate (2.5 mM each) were used as substrates.

780 Mitochondrial preparations (0.25 mg protein. mL^{-1}) and 3-hydroxytetradecanoic (3HTA) or

781 3-hydroxypalmitic (3HPA) acids (10-100 μM) were added to the incubation medium in the

782 beginning of the assays. Controls were performed in the absence of fatty acids. Values are

783 means ± standard deviation for three to five independent experiments and are expressed as

784 pmol O$_2$. s^{-1}. mg of protein^{-1}. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared to controls

785 (Duncan multiple range test).

786

787 **Fig 2. Effects of 3-hydroxypalmitic acid (3HPA) on resting (state 4) respiration in the**

788 **presence of atractyloside (ATC, 30 μM) or guanosine diphosphate (GDP, 200 μM) in**

789 **skeletal muscle mitochondria.** Glutamate plus malate (2.5 mM each) were used as

790 substrates. Mitochondrial preparations (0.25 mg protein. mL^{-1}) and 3HPA (30 μM) were

791 added to the incubation medium in the beginning of the assays. Controls were performed in

792 the absence of fatty acids. Values are means ± standard deviation for three independent

793 experiments and are expressed as pmol O_2. s^{-1}. mg of protein^{-1}. ***$P < 0.001$, compared to

794 controls (Duncan multiple range test).

795

796 **Fig 3**. **Effects of 3-hydroxypalmitic (3HPA) acids on resting (state 4), ADP-stimulated**

797 **(state 3) and uncoupled (CCCP-stimulated) respiration in skeletal muscle fibers.**

798 Glutamate (10 mM) plus malate (5 mM) were used as substrates. Approximately 8

799 milligrams of plantar skeletal muscle and 3HPA (50 e 100 μM) were added to the

800 incubation medium in the beginning of the assays. Controls were performed in the absence

801 of fatty acids. Values are means ± standard deviation for three independent experiments and

802 are expressed as pmol O_2. s^{-1}. mg of tissue^{-1}. ***$P < 0.001$, compared to controls (Duncan

803 multiple range test).

804

805 **Fig 4**. **Effects of 3-hydroxytetradecanodioic (3HTDA), 3-hydroxytetradecanoic**

806 **(3HTA) and 3-hydroxypalmitic (3HPA) acids on mitochondrial membrane potential**

807 **in Ca^{2+}-loaded skeletal muscle mitochondria.** All experiments were performed in a

808 reaction media containing mitochondrial preparations (0.5 mg protein. mL^{-1}) supported by

809 glutamate plus malate (2.5 mM each). (A) 3HTDA, 3HTA or 3HPA (30 μM, lines b-d)

810 were added 50 seconds after the beginning of the assay. (B) 3HPA (10-30 μM, lines b-d).

811 (C) 3HPA (30 µM, lines b-d). Ruthenium red (RR, 1 µM, line c) or cyclosporin A (CsA, 1

812 µM) plus ADP (300 µM) (line d) were added in the beginning of the assay. Controls (lines

813 a) were performed in the absence of fatty acids. CCCP was added at the end of the assays.

814 Traces are representative of three independent experiments and were expressed as

815 fluorescence arbitrary units (FAU). $**P < 0.01$, $***P < 0.001$, compared to controls

816 (Duncan multiple range test).

817

818 **Fig 5**. **Effects of 3-hydroxytetradecanodioic (3HTDA), 3-hydroxytetradecanoic**

819 **(3HTA) and 3-hydroxypalmitic (3HPA) acids on mitochondrial matrix NAD(P)H**

820 **content in Ca^{2+}-loaded skeletal muscle mitochondria.** All experiments were performed

821 in a reaction media containing mitochondrial preparations (0.5 mg protein. mL^{-1}) supported

822 by glutamate plus malate (2.5 mM each). 3HTDA, 3HTA or 3HPA (30 µM, lines b-d) were

823 added 50 seconds after the beginning of the assay. CCCP was added at the end of the

824 assays. Traces are representative of three independent experiments and were expressed as

825 fluorescence arbitrary units (FAU). $*P < 0.05$, $**P < 0.01$, compared to controls (Duncan

826 multiple range test).

827

828 **Fig 6**. **Effects of 3-hydroxytetradecanodioic (3HTDA), 3-hydroxytetradecanoic**

829 **(3HTA) and 3-hydroxypalmitic (3HPA) acids on mitochondrial Ca^{2+} retention**

830 **capacity in Ca^{2+}-loaded skeletal muscle mitochondria.** All experiments were performed

831 in a reaction media containing mitochondrial preparations (0.5 mg protein. mL^{-1}) supported

832 by glutamate plus malate (2.5 mM each). (A) 3HTDA, 3HTA or 3HPA (30 µM, lines b-d)

833 were added 50 seconds after the beginning of the assay. (B) 3HPA (10-30 µM, lines b-d).

834   (C) 3HPA (30 μM, lines b-d), cyclosporin A (CsA, 1 μM) (line d) were added in the

835   beginning of the assays. Controls (lines a) were performed in the absence of fatty acids.

836   CCCP was added at the end of the assays. Traces are representative of three independent

837   experiments and were expressed as fluorescence arbitrary units (FAU).

838

839   **Fig 7. Effects of 3-hydroxypalmitic acid (3HPA) on mitochondrial membrane fluidity**

840   **in skeletal muscle mitochondria.** Glutamate plus malate (2.5 mM each) were used as

841   substrates. Mitochondrial preparations (0.5 mg protein. mL^{-1}) and 3-hydroxypalmitic acid

842   (3HPA, 50 μM) were added to the incubation medium in the beginning of the assays. Five

843   minutes afterwards, laurdan (1 μM) was added to the reaction medium and incubated for 30

844   minutes. Controls were performed in the absence of 3HPA. Values are means ± standard

845   deviation for four independent experiments and are expressed as generalized polarization

846   (GP). *$P < 0.05$, compared to controls (Duncan multiple range test).

847

848

849

Figure 1
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State 4 respiration
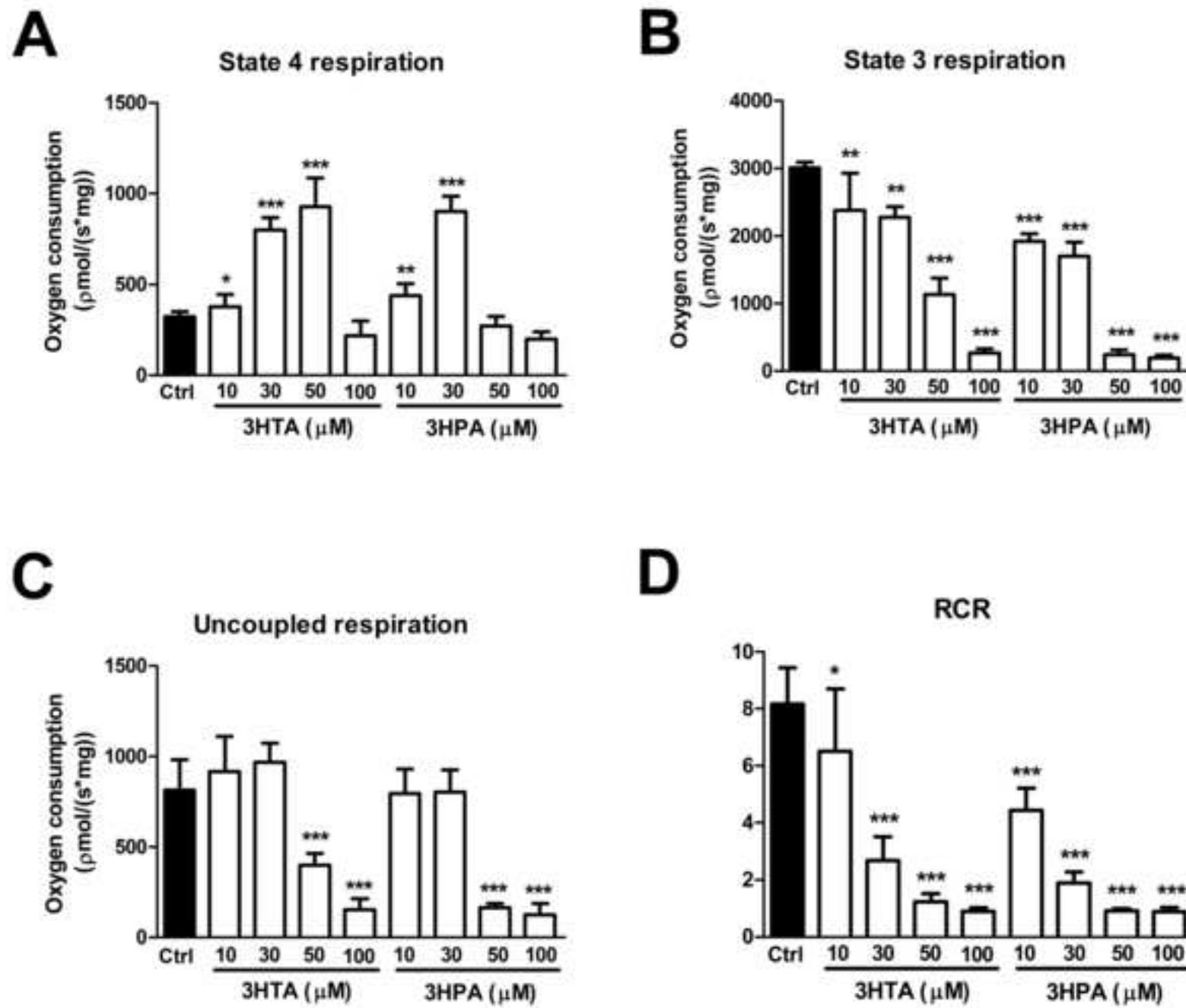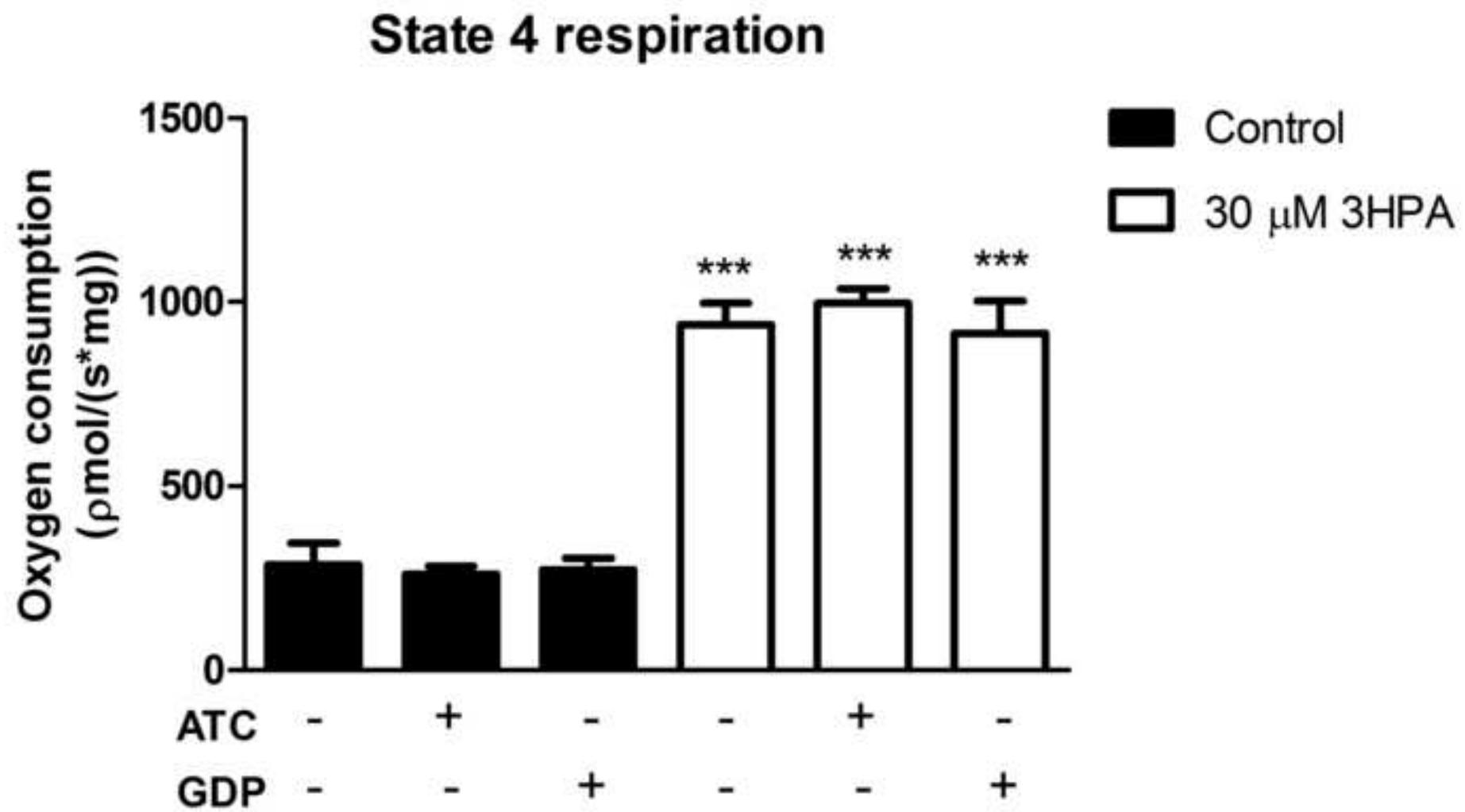
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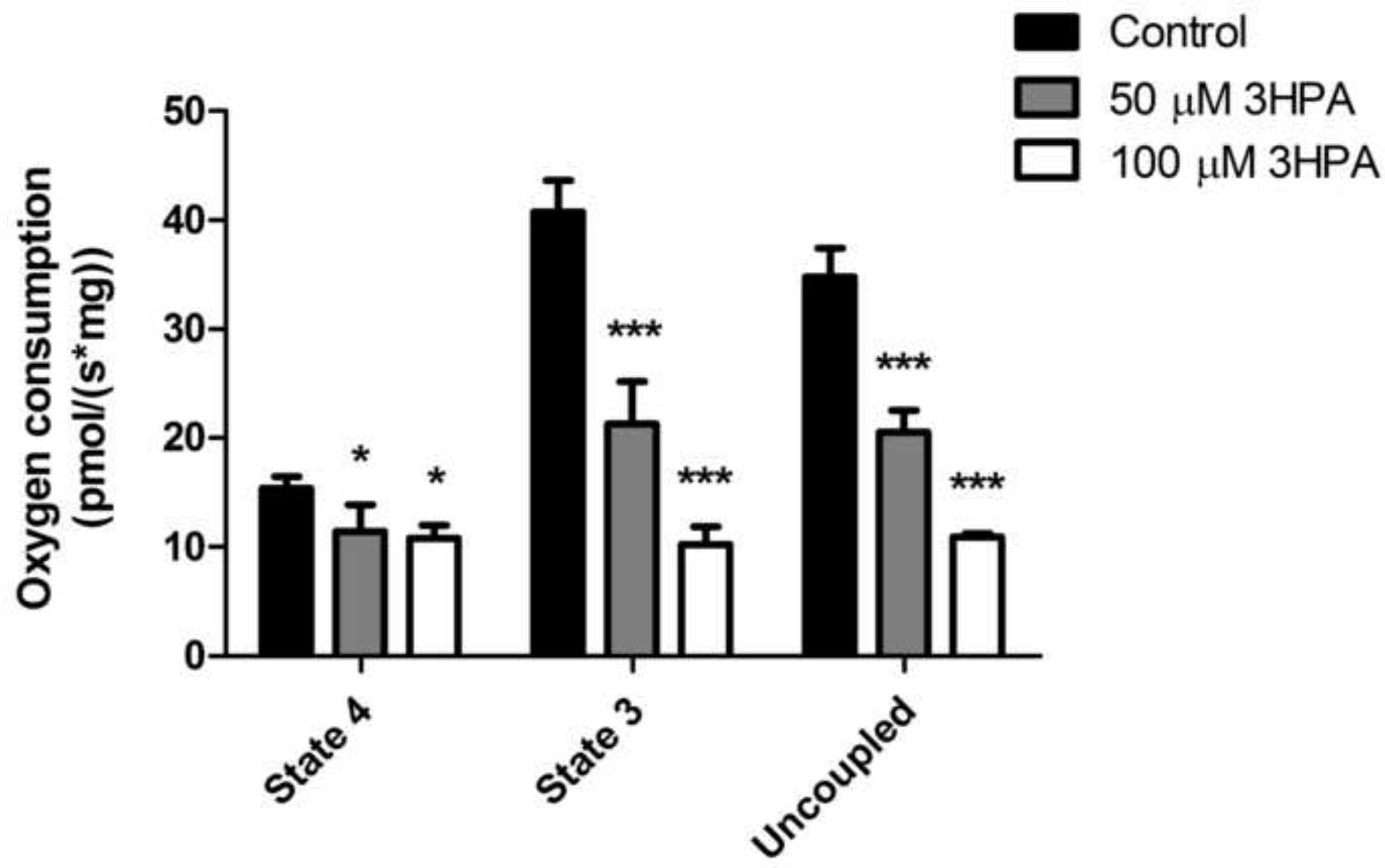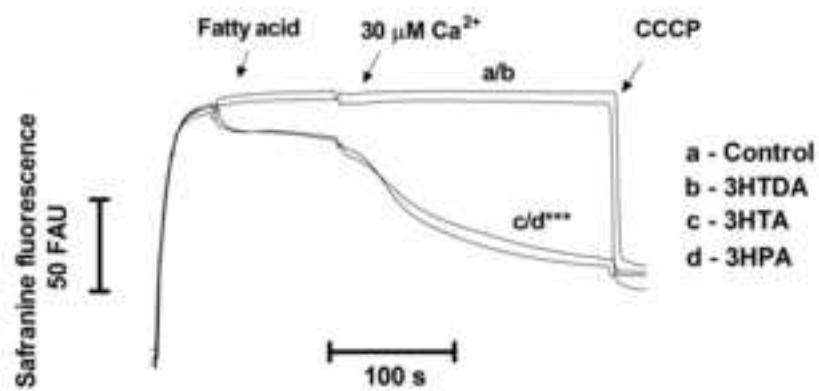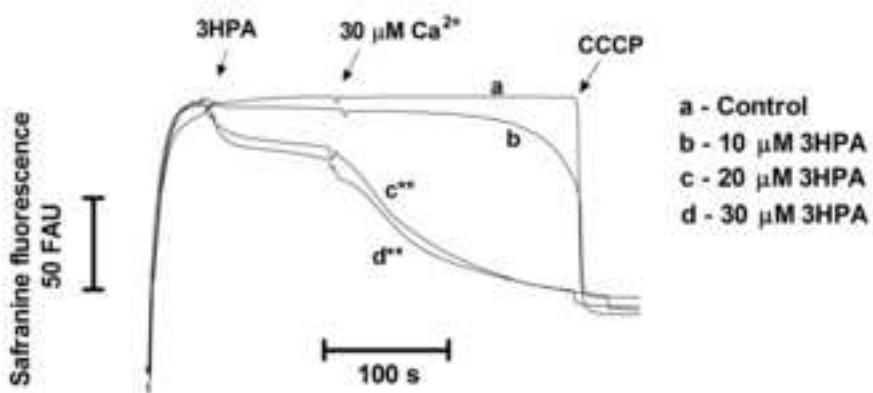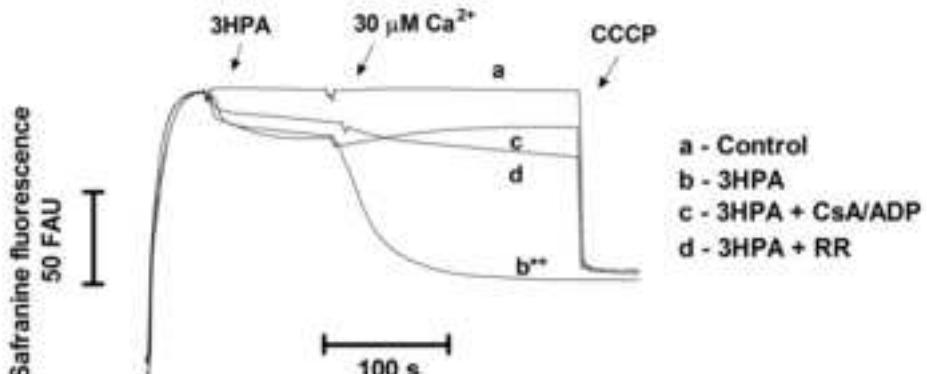
Figure 5
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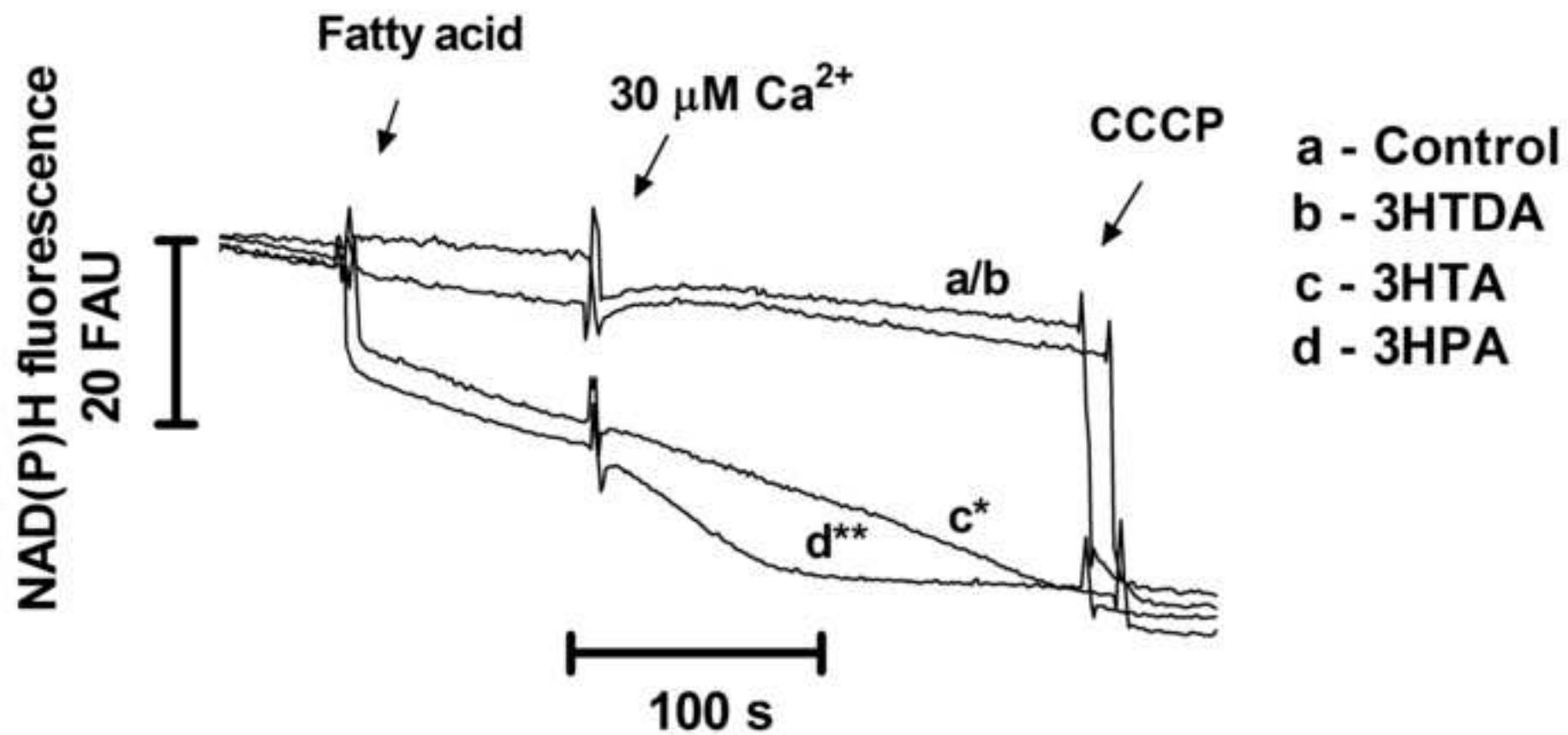
Figure 5

Figure 6
**Click here to download high resolution image**
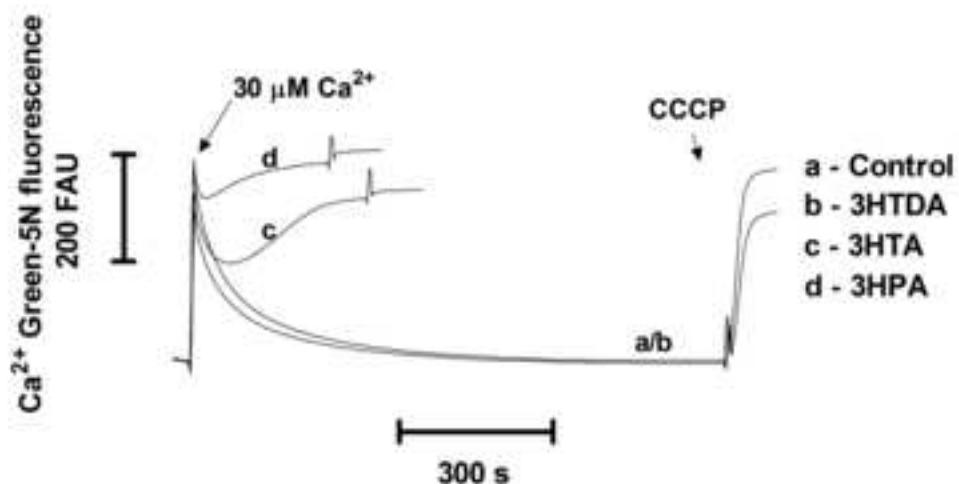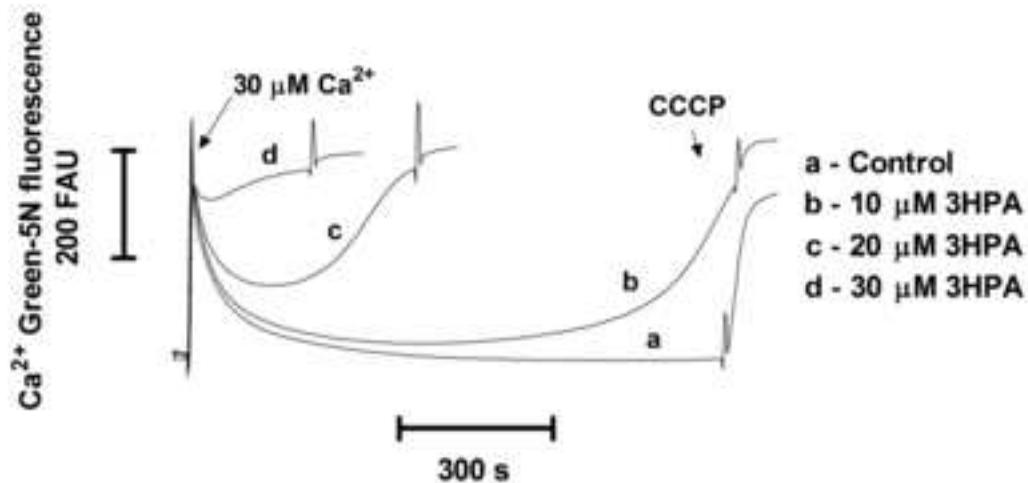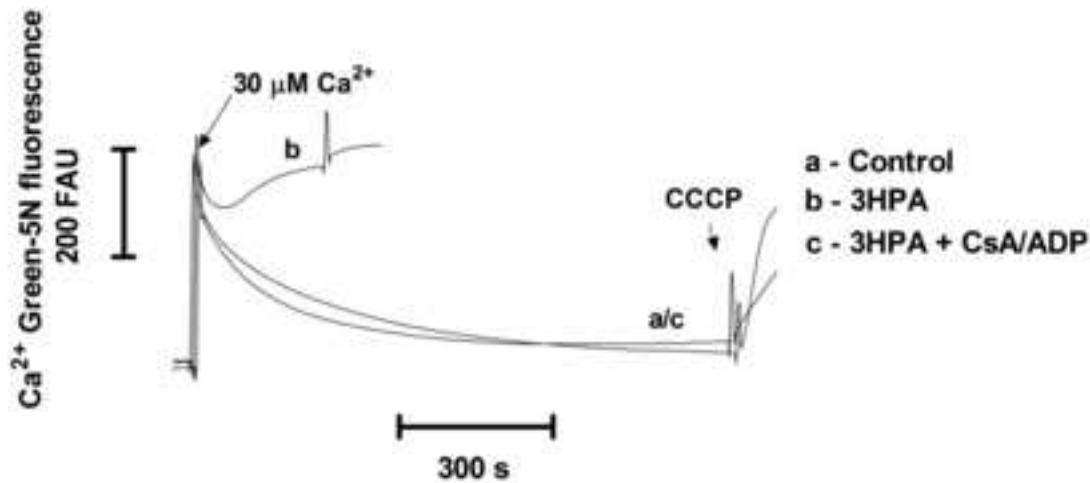
Figure 6

Figure 7
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Mitochondrial Membrane Fluidity
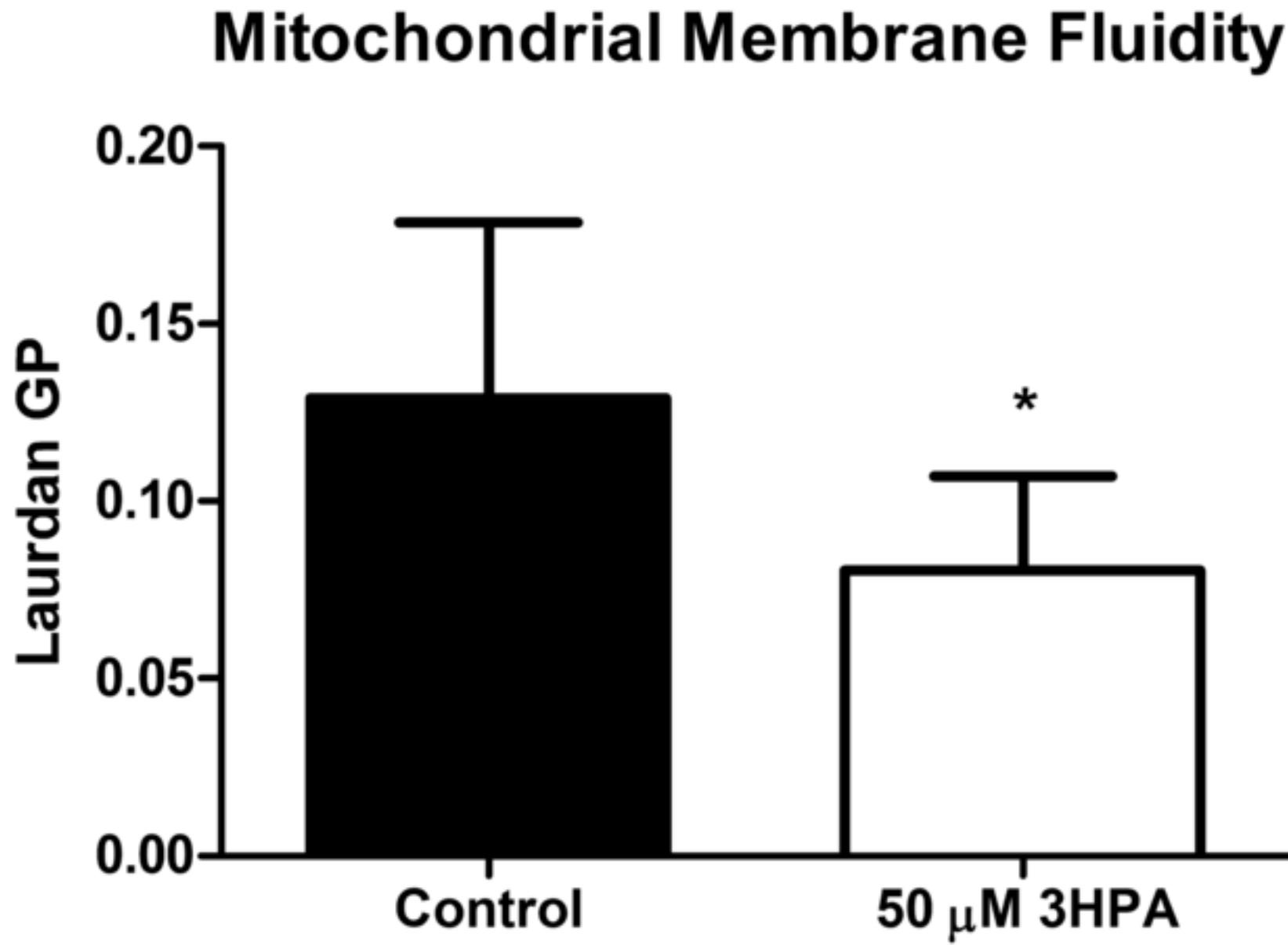
Discussão e Conclusões

3. DISCUSSÃO

Pacientes afetados pelas deficiências da MTP e da LCHAD apresentam sintomatologia clínica variada, incluindo cardiomiopatia aguda, insuficiência hepática, fraqueza muscular, episódios recorrentes de rabdomiólise, convulsão, letargia e retinopatia (Onkenhout, Venizelos et al. 1995; Rinaldo, Raymond et al. 1998; Spiekerkoetter, Bennett et al. 2004; Roe and Ding 2014). Na forma clínica mais grave dessas doenças, os pacientes desenvolvem cardiomiopatia aguda severa usualmente desencadeada por jejum ou crises de descompensação metabólica com catabolismo acelerado, podendo resultar no óbito dos indivíduos afetados (Sykut-Cegielska, Gradowska et al. 2011). Apesar dos pacientes apresentarem rabdomiólise, os mecanismos moleculares responsáveis pelas alterações no músculo cardíaco e esquelético ainda não são completamente conhecidos. Neste sentido, a deficiência energética decorrente de um bloqueio na β-oxidação com falta na produção de ATP, hiperamonemia e toxicidade dos metabólitos acumulados tem sido propostos como possíveis mecanismos das manifestações clínicas e alterações estruturais anatômicas em vários tecidos dos pacientes afetados por esses distúrbios (Spiekerkoetter and Wood 2010; Olpin 2013).

Deve-se ressaltar a importância da oxidação de ácidos graxos para os músculos cardíaco e esquelético, sendo uma fundamental fonte de ATP para esses tecidos. Durante situações de catabolismo elevado, tais como jejum prolongado e processos infecciosos acompanhados de inflamação, ocorre o acúmulo dos metabólitos precursores da β-oxidação de ácidos graxos de cadeia longa, particularmente os 3HTA e 3HPA, em vários tecidos, incluindo os tecidos musculares dos pacientes com as deficiências da MTP e LCHAD (Graber, Sumida et al. 1994).

Durante crises de descompensação metabólica, os pacientes afetados também apresentam hipoglicemia hipocetótica e um aumento na concentração plasmática do ácido lático, de transaminases e da creatina cinase. Essas alterações bioquímicas são características de lesões hepáticas e musculares associadas com uma disfunção mitocondrial. Neste particular, estudos

anteriores demonstraram que os principais 3-hidroxiácidos acumulados nessas doenças, 3HTA e 3HPA, comprometem funções mitocondriais especialmente em cérebro e fígado de ratos (Tonin, Amaral et al. 2013; Hickmann, Cecatto et al. 2015), mas pouco se sabe a respeito da toxicidade desses metabólitos sobre tecidos musculares.

No presente trabalho observamos que os principais LCHFA acumulados nas deficiências da MTP e LCHAD (3HTA e 3HPA) se comportam como desacopladores, inibidores metabólicos e indutores do PTP, além de comprometerem a retenção de cálcio mitocondrial comprometendo dessa maneira a homeostase energética em coração e músculo esquelético de ratos. Por outro lado, o análogo dicarboxílico do 3HTA, 3HTDA que também se acumula nessas doenças, não alterou nenhum dos parâmetros analisados, indicando uma seletividade para os ácidos monocarboxílicos.

Inicialmente, demonstramos que o 3HPA e 3HTA aumentaram o consumo de oxigênio no estado 4 da respiração mitocondrial e diminuíram o RCR em preparações mitocondriais obtidas de músculo esquelético, quando em concentrações baixas (10-30 μM), indicando um comportamento desacoplador. Curiosamente, esses compostos provocaram um efeito oposto de inibição do estado 4 nas maiores concentrações testadas (50-100 μM), o qual é característico dos inibidores metabólicos. Neste particular, 3HTA e 3HPA diminuíram marcadamente o consumo de oxigênio no estado 3 (estimulado por ADP) e desacoplado (estimulado por CCCP) da respiração mitocondrial, corroborando com a hipótese de que esses metabólitos agem também como potentes inibidores metabólicos em mitocôndrias utilizando glutamato/malato como substrato.

Ácidos graxos de cadeia longa são classicamente descritos como desacopladores da fosforilação oxidativa. O mecanismo desse efeito se deve ao seu transporte na forma não dissociada para a matriz mitocondrial pela bicamada lipídica da membrana mitocondrial e pelo efluxo mitocondrial na forma dissociada por difusão facilitada por proteínas, como ANT e UCP (Schonfeld, Schild et al. 1989; Schonfeld 1992). Substâncias inibidoras destas duas proteínas podem ser utilizadas com o intuito de desvendar os

mecanismos envolvidos. Estudos demonstraram que o ATC, um inibidor do ANT, previne em parte o efeito desacoplador induzido por ácido palmítico em mitocôndrias de músculo esquelético de ratos (Mokhova and Khailova 2005) e também que o GDP prejudica o transporte de ácidos graxos pela UCP (Goglia and Skulachev 2003; Affourtit, Crichton et al. 2007). No entanto, nossos resultados não indicam o envolvimento dessas proteínas, uma vez que o aumento do consumo de oxigênio provocado pelo 3HPA em mitocôndrias de músculo esquelético não foi alterado por ATC ou GDP. Assim, observamos que os ácidos graxos de cadeia longa 3-hidroxilados desacoplam a oxidação fosforilativa por um mecanismo diferente dos não hidroxilados. Não podemos descartar que outros transportadores mitocondriais possam estar envolvidos nesse efeito, tais como o trocador glutamato/aspartato (Skulachev 1998; Mokhova and Khailova 2005) e o transportador de fosfato (Zackova, Kramer et al. 2000). O efeito desacoplador da fosforilação oxidativa induzido pelo 3HTA e 3HPA também pode estar relacionado com o aumento da fluidez das membranas mitocondriais (Isaacson and Van Thiel 1988; Zhao and Hirst 1990; Schroder, Rathner et al. 1996) que demonstramos ser induzido pelo 3HPA, através da fluorescência do laurdan, em mitocôndrias de músculo esquelético.

Verificamos também que o 3HPA é capaz de agir como um forte inibidor metabólico em fibras musculares permeabilizadas, realçando a relevância fisiopatológica de nossos achados, uma vez que essa abordagem para medir o consumo de oxigênio *in situ* permite uma análise da função mitocondrial dentro de um sistema celular integrado, na sua posição citosólica normal, preservando interações essenciais com o citoesqueleto, núcleo e retículo endoplasmático (Kuznetsov, Veksler et al. 2008).

As alterações mitocondriais provocadas pelo 3HTA e 3HPA podem estar relacionadas com a acidemia lática e com as anormalidades mitocondriais observadas nos pacientes com deficiência da LCHAD (Amirkhan, Timmons et al. 1997; Tyni and Pihko 1999). A alta demanda energética para a contração do músculo cardíaco e esquelético tornam esses tecidos extremamente dependentes do seu metabolismo oxidativo mitocondrial, fazendo com que os efeitos provocados pelo 3HTA e 3HPA tenham potencial para serem comprometedores nesses tecidos.

Além disso, verificamos que baixas concentrações (10-30 µM) do 3HTA e 3HPA diminuíram marcadamente o $\Delta\Psi m$ em mitocôndrias de músculo esquelético e coração na presença de Ca^{2+} exógeno. Considerando que esse efeito foi prevenido pelo inibidor do transportador uniporte de Ca^{2+}, rutênio vermelho (RR) (Moore 1971) e pela combinação de ciclosporina A (CsA) e ADP, inibidores do PTP via inativação da ciclofilina D (uma das proteínas componentes do PTP) e por interagir com o ANT, respectivamente, esses resultados indicam o envolvimento do PTP nos efeitos provocados pelo 3HTA e 3HPA na presença de Ca^{2+}.

A abertura do PTP e a consequente permeabilização não-seletiva da mitocôndria podem induzir inchamento mitocondrial, a liberação de Ca^{2+}, Mg^{2+}, glutationa, NADH e NADPH, bem como de fatores pró-apoptóticos, como o citocromo c, comprometendo drasticamente a fosforilação oxidativa e síntese de ATP e, finalmente, provocando apoptose ou necrose (Zoratti and Szabo 1995; Crompton, Virji et al. 1999; Starkov 2010; Rasola and Bernardi 2011; Bernardi and von Stockum 2012; Rizzuto, De Stefani et al. 2012; Figueira, Barros et al. 2013). Neste contexto, também observamos que o 3HTA e 3HPA diminuíram o conteúdo de NAD(P)H e induziram inchamento mitocondrial na presença de Ca^{2+}, os quais também foram prevenidos por RR e CsA/ADP, contribuindo para a hipótese de que os ácidos graxos hidroxilados de cadeia longa (LCHFA) também agem como indutores do PTP. A diminuição do conteúdo de NAD(P)H pode ser explicada pela perda dos equivalentes reduzidos devido à permeabilidade não seletiva induzida pela abertura do PTP, bem como pelo alto consumo de NADH devido à ativação do fluxo de elétrons pela cadeia respiratória (Le-Quoc and Le-Quoc 1989; Kehrer and Lund 1994; Kowaltowski, Castilho et al. 2001). Finalmente, a redução significativa na síntese de ATP em coração provocada pelo 3HTA e 3HPA na presença de Ca^{2+} indica que os efeitos causados por esses compostos sobre a mitocôndria se refletem na síntese de ATP mitocondrial, comprometendo sobremaneira a homeostase energética celular.

Também verificamos em nosso estudo que os ácidos graxos estudados reduziram a capacidade mitocondrial de captação e retenção de Ca^{2+} em

coração e músculo esquelético de ratos que é uma função crucial da mitocôndria no tamponamento do Ca^{2+} citosólico em células musculares durante os processos de contração muscular (Yi, Ma et al. 2011; Drago, De Stefani et al. 2012). Esse efeito foi uma vez mais totalmente prevenido por CsA, indicando o envolvimento do PTP no mesmo. Deve-se notar que as concentrações celulares de Ca^{2+} são críticas para o funcionamento normal dos cardiomiócitos durante a contração cardíaca, sugerindo que a homeostase mitocondrial do Ca^{2+} como um importante mecanismo regulatório da fisiologia do coração que pode ser afetada em estados patológicos (Drago, De Stefani et al. 2012). Além disso, considerando que um desequilíbrio na concentração intracelular de Ca^{2+} tem sido sugerido como um evento fisiopatológico que pode levar à rabdomiólise (Hamel, Mamoune et al. 2015), é possível que uma indução do PTP pode indiretamente representar um potencial mecanismo envolvido nas lesões musculares associadas a essas manifestações.

É importante destacar que também utilizamos em nossos experimentos o análogo dicarboxílico do 3HTA, o 3HTDA, e que este não foi capaz de alterar nenhum dos parâmetros estudados, mostrando então uma especificidade dos efeitos aos ácidos monocarboxílicos que mais se acumulam na deficiência da LCHAD e MTP. Uma possível explicação para isso pode ser porque o 3HTDA é mais polar que os monocarboxílicos e por isso apresenta maior dificuldade de cruzar a membrana mitocondrial interna por difusão. Entretanto, mais estudos são necessários para explicar o motivo pelo qual ele não altera os parâmetros estudados.

Por outro lado, a indução do PTP provocado por 3HTA e 3HPA pode dever-se ao desacoplamento da fosforilação oxidativa como previamente demonstrado (Kowaltowski, Castilho et al. 1996; Castilho, Vicente et al. 1997; Zago, Castilho et al. 2000; Bernardi and von Stockum 2012).

Nossos achados em músculo esquelético também mostraram que o 3HPA provoca um aumento na fluidez da membrana mitocondrial. Neste particular, esse mecanismo tem sido associado à abertura do PTP por estar relacionado a alterações conformacionais das proteínas que formam o poro (Ricchelli, Gobbo et al. 1999). Dessa maneira, um mecanismo alternativo para

explicar a abertura do PTP provocado pelo 3HPA poderia ser um aumento na fluidez da membrana mitocondrial, fato que foi observado em músculo esquelético independente da presença de Ca^{2+}, caracterizando um evento anterior à indução do PTP. Ainda, a contrapartida também é verdadeira, pois foi descrito que a redução da fluidez de membrana mitocondrial deixa essa organela mais resistente à abertura do PTP (Colell, Garcia-Ruiz et al. 2003).

Estudos prévios do nosso laboratório demonstraram que os principais ácidos graxos acumulados nas deficiências da MTP e LCHAD são capazes de comprometer a homeostase energética e a capacidade de retenção de cálcio em mitocôndrias de cérebro e fígado (Tonin, Amaral et al. 2013; Hickmann, Cecatto et al. 2015). No entanto, os resultados expostos nessa dissertação mostraram que os efeitos provocados pelo 3HTA e 3HPA sobre as funções mitocondriais se mostraram mais intensos em mitocôndrias obtidas de tecidos musculares. Dessa maneira, podemos especular que a maior vulnerabilidade demonstrada pelas mitocôndrias de coração e músculo esquelético de ratos quando comparadas às de fígado, mas especialmente às de cérebro, pode estar associada aos graves sintomas cardíacos que inclusive podem levar o paciente ao óbito, bem como com as lesões musculares associadas a episódios de rabdomiólise que frequentemente acometem esses pacientes (Rocchiccioli, Wanders et al. 1990; Amirkhan, Timmons et al. 1997; Tyni, Rapola et al. 1997; Moczulski, Majak et al. 2009). Além disso, deve-se considerar o fato de que tecidos musculares são altamente dependentes da oxidação de ácidos graxos para obter suas necessidades energéticas, especialmente em períodos de jejum e estresse catabólico, fazendo com a expressão das enzimas necessárias para a oxidação de ácidos graxos seja maior nesses tecidos. Daí a possibilidade de um maior acúmulo intracelular dos metabólitos acumulados quando ocorre bloqueio das rotas de oxidação nas deficiências da MTP e LCHAD. Finalmente, as alterações mitocondriais provocadas pelo 3HTA e 3HPA sobre o coração e músculo esquelético, tais como a abertura do PTP, já foram relacionadas ao dano cardíaco provocado pela reperfusão após quadros de isquemia, e, além disso, a inibição da abertura pela CsA mostrou-se cardioprotetora, bem como com os danos relacionados a rabdomiólise ocasionada por oclusão arterial do membro

inferior, os quais foram diminuídos pelo uso de NIM-811, análogo da CsA (Garbaisz, Turoczi et al. 2014; Ong, Dongworth et al. 2014; Halestrap and Richardson 2015; Ong, Samangouei et al. 2015).

Outra questão que deve ser enfatizada, especialmente analisando nossas condições experimentais, é a maior capacidade oxidativa e de retenção de cálcio no coração e músculo esquelético, bem como uma menor sensibilidade à indução do PTP dependente de Ca^{2+}, quando comparados ao fígado e cérebro, tornando mais impactante a vulnerabilidade de mitocôndrias de tecidos musculares frente à toxicidade do 3HTA e 3HPA sobre essas funções mitocondriais.

Neste trabalho foi demonstrado que mesmo baixas concentrações (10-30 µM) de ácidos graxos monocarboxílicos hidroxilados são capazes de alterar a homeostase mitocondrial, agindo como desacopladores, inibidores metabólicos e indutores do PTP. É importante ressaltar que essas concentrações são similares ou ainda menores do que as concentrações plasmáticas apresentadas por pacientes com as deficiências da MTP e LCHAD durante crises de descompensação metabólica, caracterizadas por uma piora significativa nos sintomas cardíacos e musculares (Hagenfeldt, von Dobeln et al. 1990; da Costa Ferreira, Schuck et al. 2008). Essas crises são geralmente secundárias a infecções e/ou jejum, levando a um acelerado catabolismo que aumenta a quantidade de ácidos graxos circulantes e nos tecidos que possuem uma alta taxa de oxidação de ácidos graxos (Hagenfeldt, von Dobeln et al. 1990).

Finalmente, observamos que o 3HTA e o 3HPA provocaram efeitos semelhantes, comprometendo a bioenergética mitocondrial em fibras de músculo esquelético de ratos. Tais resultados obtidos em tecido íntegro validam aqueles obtidos em mitocôndrias isoladas.

Concluindo, foi apresentado neste trabalho pela primeira vez que os principais metabólitos acumulados na deficiência da LCHAD agem como desacopladores, inibidores metabólicos, indutores do PTP e prejudicam a capacidade de retenção de cálcio em mitocôndrias obtidas de músculo cardíaco e esquelético, bem como em fibras musculares. Verificamos ainda

que os efeitos provocados por esses compostos se refletem numa drástica redução na produção de ATP mitocondrial, caracterizando um comprometimento da homeostase energética. Neste sentido, é provável que um forte comprometimento das funções mitocondriais provocados pelos principais metabólitos acumulados nas deficiências da MTP e LCHAD esteja associado aos sintomas cardíacos, bem como à atrofia e sintomas musculares apresentados pelos pacientes afetados por essas doenças.

4. CONCLUSÕES

- O 3HTA e o 3HPA comprometem a respiração mitocondrial, aumentando o estado 4 em baixas concentrações e diminuindo o RCR, utilizando glutamato/malato como substrato em mitocôndrias de músculo cardíaco e esquelético de ratos jovens, sugerindo um efeito desacoplador destes metabólitos. Em contrapartida, em maiores concentrações esses ácidos foram capazes de diminuir o estado 4 o que, juntamente com a diminuição do estado 3 e da respiração desacoplada, está relacionado com a inibição metabólica que eles causam.

- O atractilosídeo e o GDP não foram capazes de interferir no aumento do estado 4 da respiração causados pelo 3HPA em mitocôndrias de músculo esquelético, indicando que uma interação entre esse composto com o ANT e UCP, respectivamente, não está envolvida nos efeitos deletérios provocados pelos LCHFA.

- 3HTA e 3HPA em baixas concentrações (10-30 μM), mas não o 3HTDA, dissiparam o $\Delta\Psi m$ na presença de Ca^{2+} em mitocôndrias de músculo cardíaco e esquelético. RR e CsA mais ADP preveniram esse efeito, enfatizando o envolvimento do Ca^{2+}.

- 3HTA e 3HPA em baixas concentrações (30 μM) também diminuíram o conteúdo de NAD(P)H na presença de Ca^{2+}, em mitocôndrias de músculo cardíaco e esquelético. 3HTDA não alterou este parâmetro.

- 30 μM de 3HTA e 3HPA, mas não de 3HTDA, também provocaram inchamento mitocondrial na presença de Ca^{2+} em mitocôndrias de músculo cardíaco. RR e CsA mais ADP foram capazes de prevenir este efeito.

- 3HTA e 3HPA (10-30 μM) reduziram significativamente a capacidade de retenção de cálcio mitocondrial em mitocôndrias de músculo cardíaco e esquelético, efeito que foi prevenido por CsA mais ADP. 3HTDA não alterou este parâmetro.

- 3HPA e 3HTA (20 μM) diminuíram drasticamente a produção de ATP, na presença de Ca^{2+} em mitocôndrias de músculo cardíaco.

- 3HPA (50 µM) aumentou a fluidez de membrana mitocondrial em mitocôndrias de músculo esquelético.

- 3HPA (50-100 µM) também prejudicou a bioenergética mitocondrial em fibras de músculo esquelético, validando os resultados obtidos em mitocôndrias isoladas.

5. PERSPECTIVAS

- Verificar a influência do 3HTA e 3HPA sobre a viabilidade, morfologia, bioenergética mitocondrial e homeostase do cálcio em culturas de cardiomiócitos.

- Verificar a influência do 3HTA e 3HPA sobre a viabilidade, morfologia, bioenergética mitocondrial e homeostase do cálcio em culturas de miócitos.

- Investigar o papel do PTP mitocondrial nos efeitos provocados pelos LCHFA em culturas de cardiomiócitos e miócitos utilizando CsA como agente protetor.

- Verificar a influência do 3HTA e 3HPA sobre parâmetros importantes da homeostase redox em fígado e nos tecidos musculares cardíaco e esquelético.

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