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Cristiane Cecatto

EFEITOS DOS PRINCIPAIS ÁCIDOS GRAXOS ACUMULADOS NA DEFICIÊNCIA DA DESIDROGENASE DE ACILAS-COA DE CADEIA MUITO LONGA SOBRE IMPORTANTES FUNÇÕES MITOCONDRIAIS EM CORAÇÃO, FÍGADO E MÚSCULO ESQUELÉTICO

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutora em Bioquímica.

Orientador: Prof. Dr. Moacir Wajner

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2019

Para minha família e para todas as pessoas que acreditam na ciência

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"Happiness can be found, even in the darkest of times, if one only remembers to turn on the light."

Albus Dumbledore

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RESUMO

As doenças de oxidação mitocondrial de ácidos graxos são patologias genéticas reconhecidas como importantes causas de morbidade e mortalidade na população infanto-juvenil. Uma das mais comuns é a deficiência da desidrogenase de acilas-CoA de cadeia muito longa (VLCAD). Essa doença é caracterizada bioquimicamente pelo acúmulo de ácidos graxos de cadeia longa e de seus conjugados de carnitina nos tecidos e líquidos biológicos dos pacientes afetados e, clinicamente, por disfunção cardíaca, hepática e muscular. Os ácidos cis-5-tetradecenoico (Cis-5) e mirístico (Myr) são os que mais se acumulam nessa doença, sendo que o primeiro é o marcador bioquímico. As disfunções cardíaca e hepática, que podem levar os pacientes à morte, bem como os recorrentes episódios de rabdomiólise ainda não possuem sua fisiopatologia esclarecida. A presente investigação objetivou estudar os efeitos desses metabólitos acumulados sobre parâmetros importantes da função mitocondrial (parâmetros respiratórios, potencial de membrana ($\Delta\Psi m$), conteúdo de NAD(P)H, inchamento mitocondrial, capacidade de retenção de Ca^{2+}, liberação de citocromo *c*, produção de ATP, atividades dos complexos da cadeia respiratória e enzimas do ciclo do ácido cítrico) em mitocôndrias isoladas de coração, fígado e músculo esquelético de ratos jovens, bem como em fibras permeabilizadas de coração e de músculo esquelético e células hepáticas e cardíacas cultivadas. Verificamos que o Cis-5 e o Myr, em concentrações patológicas, reduziram significativamente os estados 3 e desacoplado da respiração celular, a produção de ATP, o $\Delta\Psi m$ e a capacidade de retenção de Ca^{2+} nos três tecidos testados, bem como o conteúdo de NAD(P)H na matriz mitocondrial no coração, e a atividade do complexo I no coração, do complexo I-III no fígado e músculo esquelético e da enzima α-cetoglutarato desidrogenase no fígado. Estes ácidos graxos também aumentaram o estado 4 da respiração celular (efeito desacoplador) em todos os tecidos testados, com o envolvimento do translocador de nucleotídeos de adenina em coração e fígado, pois o carboxiatractilosídeo atenuou significantemente o aumento do estado 4 provocado pelos Cis-5 e Myr. Além disso, o Cis-5 e o Myr induziram inchamento mitocondrial e a liberação de citocromo *c* no fígado. Observamos ainda que os inibidores clássicos da formação do poro de transição de permeabilidade mitocondrial (MPT) ciclosporina A mais ADP, bem como o inibidor da captação de Ca^{2+} rutênio vermelho preveniram completamente a diminuição do $\Delta\Psi m$ mitocondrial em todos os tecidos testados, bem como a indução do inchamento mitocondrial em fígado, provocados pelo Myr e Cis-5 na presença de Ca^{2+}, sugerindo, respectivamente, que a indução da abertura do poro de MTP e a contribuição do Ca^{2+} estão envolvidas nesses efeitos. Os efeitos observados na respiração celular obtidos em mitocôndrias isoladas se confirmaram em preparações *in situ* de células cultivadas (cardiomiócitos e hepatócitos) e em fibras musculares cardíacas e esqueléticas, reforçando os efeitos deletérios desses ácidos graxos. Não foram observadas alterações na homeostase redox avaliados através do conteúdo de espécies reativas ao ácido tiobarbitúrico e glutationa reduzida em coração. Os achados no presente trabalho indicam que, em concentrações patológicas, os ácidos graxos de cadeia longa que mais se acumulam na deficiência da VLCAD prejudicam a bioenergética mitocondrial e a homeostase do Ca^{2+}, atuando como desacopladores e inibidores metabólicos da fosforilação oxidativa, além de serem indutores da abertura do poro de MPT em coração, fígado e músculo esquelético. Assim, presumimos que o comprometimento na bioenergética e na homeostase do Ca^{2+} possam contribuir para as manifestações cardíacas, hepáticas e musculares observadas na deficiência da VLCAD.

Palavras-chave: deficiência da desidrogenase de acilas-CoA de cadeia muito longa; mitocôndria; bioenergética; coração; fígado; músculo esquelético.

ABSTRACT

The genetic fatty acid oxidation disorders are genetic pathologies recognized as important causes of morbidity and mortality in child-juvenile population. One of the most common is very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency. This disease is biochemically characterized by accumulation of fatty acids and their correspondent carnitine derivatives in tissues and biological fluids from affected patients, and clinically by cardiac, hepatic and muscular dysfunction. Cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids are those that most accumulate in this disorder, wherein the first one is the biochemical hallmark. The cardiac and hepatic dysfunction, which may lead the patients to death, as well as the recurrent rhabdomyolysis episodes have the pathogenesis still poorly unknown. The present investigation was aimed to study the effects of these accumulated metabolites on important mitochondrial function parameters (respiratory parameters, membrane potential ($\Delta\Psi m$), NAD(P)H content, mitochondrial swelling, Ca^{2+} retention capacity, cytochrome *c* release, ATP production, respiratory chain complexes and citric acid cycle enzymes activities) in isolated mitochondria of heart, liver and skeletal muscle from young rats, as well as in permeabilized heart and skeletal muscle fibers and hepatic and cardiac cultivated cells. We verified that Cis-5 and Myr, at pathological concentrations, strongly reduced states 3 and uncoupled of respiration, ATP production, $\Delta\Psi m$ and Ca^{2+} retention capacity in all tested tissues, as well as matrix mitochondrial NAD(P)H content in heart, complex I activity in heart, complex I-III in liver and skeletal muscle and α-ketoglutarate dehydrogenase activity in liver. Also, these fatty acids increased state 4 respiration (uncoupling effect) in all tested tissues, with the involvement of the adenine nucleotide translocator in heart and liver since carboxyatractyloside significantly attenuated the increased state 4 respiration provoked by Cis-5 and Myr. In addition, Cis-5 and Myr caused mitochondrial swelling and cytochrome *c* release in liver. We also observed that the classical inhibitors of mitochondrial permeability transition (MPT) pore cyclosporin A plus ADP, as well as the Ca^{2+} uptake blocker ruthenium red, fully prevented the decrease of $\Delta\Psi m$ in all tested tissues as well as mitochondrial swelling induction in liver provoked by Myr and Cis-5 in Ca^{2+}-loaded mitochondria, suggesting, respectively, induction of MPT pore opening and the contribution of Ca^{2+} in these effects. The effects observed in cellular respiration obtained in isolated mitochondria were confirmed in *in situ* cell preparations (cardiomyocytes and hepatocytes), as well as in cardiac and skeletal muscle fibers, reinforcing the deleterious effects of the fatty acids. In contrast, there were no alterations of redox homeostasis evaluated by the content of thiobarbituric acid reative species and reduced glutathione in heart. The present findings indicate that the major long-chain fatty acids that accumulate in VLCAD deficiency disrupt mitochondrial bioenergetics and Ca^{2+} homeostasis, acting as uncouplers and metabolic inhibitors of oxidative phosphorylation, as well as inducers of MPT pore opening in the heart, liver and skeletal muscle at pathological relevant concentrations. It is therefore presumed that disturbance of bioenergetics and Ca^{2+} homeostasis may contribute to the cardiac, hepatic and muscular manifestations observed in VLCAD deficiency.

Keywords: very-long-chain acyl-CoA dehydrogenase deficiency; mitochondria; bioenergetics; heart; liver; skeletal muscle.

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LISTA DE ABREVIATURAS

ADP – adenosina difosfato
ANT – translocador de nucleotídeos de adenina
AST – aspartato aminotransferase
ATP – adenosina trifosfato
CAC – ciclo do ácido cítrico
CAT – carboxiatractilosídeo
CCCP – cianeto de carbonila de meta-clorofenil-hidrazona
Cis-5 – ácido cis-5-tetradecenóico
CoA – coenzima A
CPT I – carnitina palmitoil transferase I
CPT II – carnitina palmitoil transferase II
CS – citrato sintase
CsA – ciclosporina A
CT – carnitina-acilcarnitina translocase
Cyp D – ciclofilina D
DGOAG – doenças genéticas de oxidação de ácidos graxos
DPC - dietilpirocarbonato
DTT – ditiotreitol
EIM – erros inatos do metabolismo
$FADH_2$ – flavina adenina dinucleotídeo reduzido
GDH – glutamato desidrogenase
GDP – guanosina difosfato
GSH – glutationa reduzida
IDH – isocitrato desidrogenase
KT – cetotiolase
LCAD – acil-CoA desidrogenase de cadeia longa
LCEH – 2-enoil-CoA hidratase de cadeia longa
LCFA – ácidos graxos de cadeia longa
LCHAD – 3-hidroxi-acil-CoA desidrogenase 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+}
MDA – malondialdeído
MDH – malato desidrogenase
mHCX – trocador H^+/ Ca^{2+}
mNCX – trocador Na^+/ Ca^{2+}
MTP – proteína trifuncional mitocondrial
Myr – ácido mirístico
NAD^+ – nicotinamida adenina dinucleotídeo
NADH – nicotinamida adenina dinucleotídeo reduzido
$NADP^+$ – nicotinamida adenina dinucleotídeo fosfato
NADPH – nicotinamida adenina dinucleotídeo fosfato reduzido
NEM – N-etilmaleimida
NNT – transidrogenase mitocondrial de nucleotídeos de nicotinamida
OXPHOS – fosforilação oxidativa

PTP – poro de transição de permeabilidade
RCR – razão de controle respiratório
RMN – ressonância magnética nuclear
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
SDH – sucinato desidrogenase
UCP – proteínas desacopladoras
VLCAD – acil-CoA desidrogenase de cadeia muito longa
VLCADD – deficiência de acil-CoA desidrogenase de cadeia muito longa
α-KGDH – α-cetoglutarato desidrogenase
ΔΨm – potencial de membrana mitocondrial

1. INTRODUÇÃO

1.1 ERROS INATOS DO METABOLISMO

Os erros inatos do metabolismo (EIM) são doenças genéticas inicialmente descritas por Archibald Garrod em 1908, sendo a primeira a alcaptonúria, um EIM do metabolismo da tirosina e fenilalanina. Essas doenças são causadas por mutações em genes que levam à deficiência funcional 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 pode, por rotas alternativas, gerar compostos que são potencialmente tóxicos (CHILDS; VALLE; JIMENEZ-SANCHEZ, 2014; SCRIVER et al., 2001) (Figura 1).

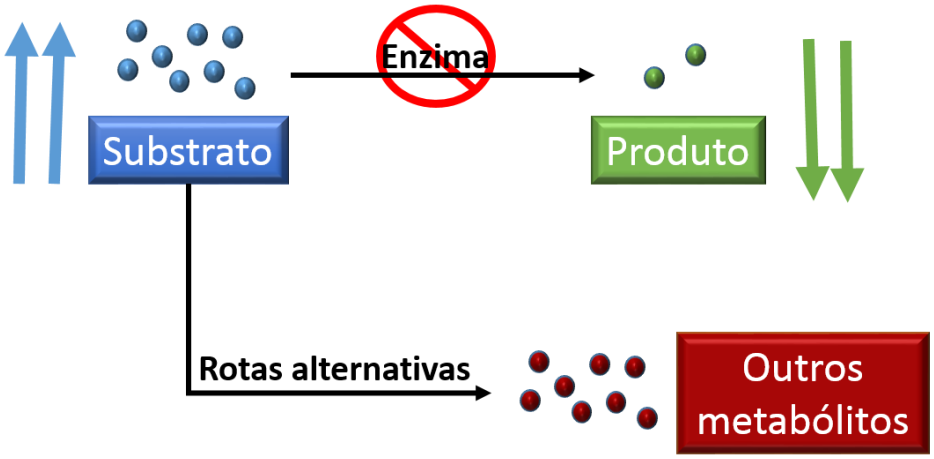

Figura 1. Esquema geral de bloqueio de uma rota metabólica que ocorre em erros inatos do metabolismo.

Apesar de serem individualmente raras, em conjunto essas doenças afetam aproximadamente um a cada 2500 recém-nascidos vivos (JEANMONOD; JEANMONOD, 2018), podendo chegar até a 1 a cada 800 recém-nascidos vivos, variando conforme a população (MAK et al., 2013; PAMPOLS, 2010). Até o momento, foram descritos mais de 1000 erros inatos do metabolismo com o defeito bioquimicamente caracterizado, a maioria deles envolvendo processos de síntese,

degradação, transporte e armazenamento de moléculas no organismo (ARNOLD, 2018). O fenótipo clínico desse grupo de doenças é heterogêneo (PAMPOLS, 2010; SCRIVER et al., 2001).

1.2 OXIDAÇÃO MITOCONDRIAL DE ÁCIDOS GRAXOS

A oxidação mitocondrial de ácidos graxos (β-oxidação) é a principal fonte de energia para os músculos esquelético e cardíaco, enquanto que no fígado se acentua especialmente durante o jejum e outras situações de estresse metabólico como em infecções, cirurgia, etc (EATON; BARTLETT; POURFARZAM, 1996). Nestas situações, a oxidação acentuada de ácidos graxos no fígado resulta na formação dos corpos cetônicos utilizados como fonte energética alternativa para órgãos extra-hepáticos, principalmente o cérebro e o coração. A β-oxidação representa, portanto, uma fonte energética importante para diversos órgãos essenciais à vida, como o coração, o cérebro, o fígado e os músculos esqueléticos (MITCHELL; FUKAO, 2014).

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 à rápida formação de acil-CoA após a entrada na célula (HOUTEN; WANDERS, 2010). Na forma de acil-CoA, eles podem ser utilizados para formação de fosfolipídios, triglicerídeos e ésteres de colesterol ou entrar na mitocôndria para sofrerem a β-oxidação. Como a membrana mitocondrial é impermeável a acil-CoA, faz-se necessária conjugação com carnitina através da carnitina palmitoiltransferase I (CPT I) localizada na membrana externa da mitocôndria, onde são convertidos a acilcarnitinas 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 palmitoiltransferase II (CPT II), regenerando a carnitina (CHAE et al., 1999).

Os acil-CoA passam pela espiral da β-oxidação liberando uma acetil-CoA, um nicotinamida adenina dinucleotídeo reduzido (NADH) e um flavina adenina dinucleotídeo reduzido ($FADH_2$) a cada ciclo (Figura 2). Esse acetil-CoA geralmente é oxidado no ciclo do ácido cítrico (CAC) a CO_2 nos tecidos. No fígado, é convertido a corpos cetônicos que são exportados para vários tecidos, tais como o cérebro e os músculos cardíaco e esquelético predominantemente em situações de estresse catabólico, como inflamação, infecções e jejum prolongado. O NADH e $FADH_2$ liberados atuam como equivalentes reduzidos para a cadeia transportadora de elétrons (NELSON; COX, 2017).

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 os ácidos graxos são oxidados por enzimas específicas conforme o comprimento da sua cadeia carbônica (curta, média ou longa). Há ainda uma proteína trifuncional mitocondrial (MTP) acoplada à membrana interna da mitocôndria que é constituída por quatro subunidades α e quatro subunidades β (CARPENTER; POLLITT; MIDDLETON, 1992). As subunidades α contêm as atividades 2-enoil-CoA hidratase de cadeia longa (LCEH) e 3-hidroxiacil-CoA desidrogenase de cadeia longa (LCHAD), e as subunidades β contém a atividade da 3-cetoacil-CoA tiolase de cadeia longa (LCKT) (JLST et al., 1996). Após algumas voltas realizadas pela MTP, o acil-CoA com a cadeia de carbonos diminuída passa a ser metabolizado na matriz mitocondrial por enzimas com afinidade aos ácidos graxos de menor número de carbonos.

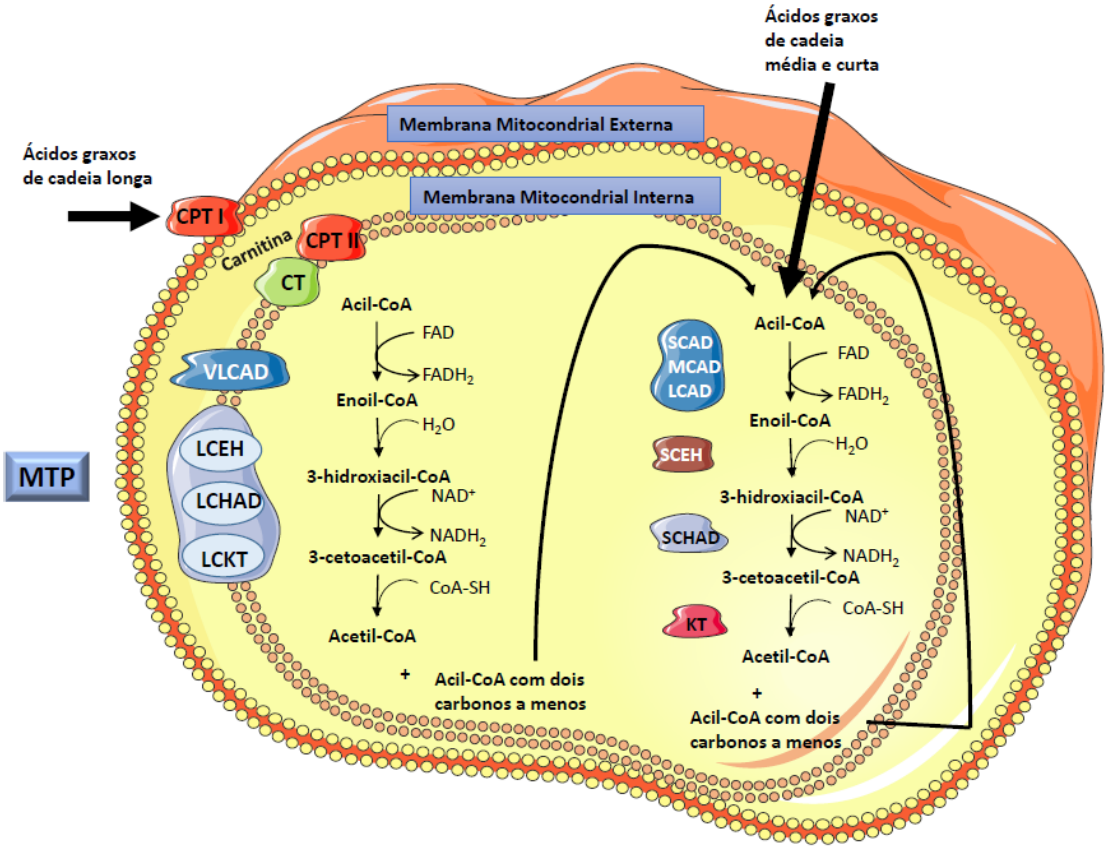

Figura 2. Esquema da β-oxidação mitocondrial de ácidos graxos. Carnitina palmitoil transferases I (CPT I) e II (CPT II); carnitina-acilcarnitina translocase (CT); acilas-CoA desidrogenase de cadeia muito longa (VLCAD); proteína trifuncional mitocondrial (MTP); 2-enoil-CoA hidratase de cadeia longa (LCEH); 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). Fonte: própria autoria.

1.3 DOENÇAS GENÉTICAS DE OXIDAÇÃO DE ÁCIDOS GRAXOS

Doenças genéticas da oxidação de ácidos graxos (DGOAG) têm sido reconhecidas nos últimos anos como importantes causas de morbidade e mortalidade, refletindo a significância fisiológica dos ácidos graxos como fonte essencial de energia para o ser humano. Atualmente já foram descritas pelo menos 17 diferentes entidades clínicas dentro deste grupo de doenças, com uma prevalência conjunta de 1 para 5000 nascimentos em algumas populações (VOCKLEY, JERRY; BENNETT; GILLINGHAM, 2014). As DGOAG podem ocorrer nas diferentes fases do metabolismo dos ácidos graxos, incluindo o ciclo da carnitina

e as reações de β-oxidação, sendo caracterizadas pelo acúmulo de ácidos graxos e seus conjugados de carnitina e glicina em tecidos e líquidos biológicos de pacientes afetados por essas doenças. O diagnóstico de pacientes suspeitos de DGOAG é, portanto, feito fundamentalmente pela quantificação de acilcarnitinas e ácidos graxos, bem como de L-carnitina, ocorrendo também aumento na excreção urinária de ácidos orgânicos dicarboxílicos e acilglicinas. A determinação das mutações envolvidas é crítica para o diagnóstico pré-natal dessas doenças e geralmente é feita quando há um ou mais casos na família. Já o diagnóstico no recém-nascido pelos testes de triagem é possível ser realizado para a grande maioria dessas doenças por espectrometria de massas em Tandem, mas é somente feito para algumas DGOAG, tais como deficiências de MCAD, VLCAD e LCHAD que seguem todos os requisitos para diagnóstico neonatal. No entanto, há uma tendência internacional de que várias outras dessas deficiências sejam incorporadas ao diagnóstico neonatal para diminuir a mortalidade e morbidade dos afetados, permitindo a implementação precoce de medidas estratégicas simples e de baixo custo, como dieta frequente e pobre em gorduras (VOCKLEY, JERRY et al., 2014).

As DGOAG cursam com uma ampla variedade de sintomas, tais como hipoglicemia, hiperamonemia, cardiomiopatia e hepatopatia neonatal e episódios recorrentes de rabdomiólise com hepatopatia iniciados na adolescência e idade adulta. A morbidade e a mortalidade são altas se o diagnóstico não é feito precocemente (VOCKLEY, JERRY et al., 2014).

1.3.1 Deficiência da desidrogenase de acilas-CoA de cadeia muito longa (VLCADD)

A desidrogenase de acilas-CoA de cadeia muito longa (VLCAD) catalisa a primeira das quatro reações sequenciais da β-oxidação de ácidos graxos de cadeia longa (C12 a C18) na mitocôndria e é codificada pelo gene *ACADVL.* A deficiência dessa enzima é um erro inato autossômico recessivo, com uma incidência de 1:30.000 a 1:100:000 (ARNOLD et al., 2009; LINDNER; HOFFMANN; MATERN, 2010; SPIEKERKOETTER et al., 2004; SPIEKERKOETTER; LINDNER; SANTER; GROTZKE; BAUMGARTNER; BOEHLES; DAS; HAASE; HENNERMANN; KARALL; DE KLERK; et al., 2009).

A apresentação clínica tem um espectro variável e os sintomas típicos geralmente aparecem após períodos de jejum ou em consequência de um processo infeccioso. Têm sido descritos três fenótipos gerais: o neonatal severo com cardiomiopatia, o infantil/adolescente com hipoglicemia hipocetótica, hepatopatia e rabdomiólise recorrente e o fenótipo adulto com somente rabdomiólise recorrente às vezes associado à hepatopatia (VOCKLEY, JERRY et al., 2014).

A forma neonatal severa é caracterizada pelo início dos sintomas muito cedo na vida, geralmente no período neonatal. Nesse grupo de pacientes a morbidade e mortalidade são altas e é comum se observar episódios recorrentes de hipoglicemia, acidose metabólica, rabdomiólise, cardiomiopatia e arritmia cardíaca. A cardiomiopatia é da forma dilatada ou hipertrófica, geralmente levando os pacientes a óbito. Aqueles que sobrevivem acabam por ter um prognóstico clínico semelhante ao fenótipo tardio, apresentando rabdomiólise recorrente na idade de 5 a 7 anos. A creatina cinase plasmática pode estar extremamente elevada e a mioglobinúria pode causar insuficiência renal necessitando hemodiálise. Os achados hepáticos são comuns, incluindo esteatose, inflamação e cirrose (GREGERSEN et al., 2001; KOMPARE; RIZZO, 2008; VOCKLEY, JERRY et al., 2014).

No fenótipo mais brando, com início dos sintomas mais tardio, ocorrem episódios de descompensação metabólica desencadeados também por febre e/ou jejum, sendo menos comum se observar cardiomiopatia, que dá lugar a hipoglicemia hipocetótica, hepatomegalia e hipotonia na infância. Conforme o paciente envelhece, a acidose metabólica e a hipoglicemia diminuem, os sintomas passam a ser dominados por dor muscular induzida por estresse ou exercício e rabdomiólise. A forma tardia, que se apresenta em adolescentes ou adultos jovens, geralmente é dominada pelos sintomas musculares e com frequência está associada com intolerância ao exercício e mioglobinúria. Os pacientes apresentam rabdomiólise recorrente, dor e fraqueza musculares que começam na adolescência e podem continuar por décadas antes do diagnóstico de deficiência da VLCAD (VLCADD) ser feito (GREGERSEN et al., 2001; KOMPARE; RIZZO, 2008; VOCKLEY, JERRY et al., 2014).

Durante as crises de descompensação metabólica observa-se um aumento significativo dos níveis dos metabólitos que tipicamente se acumulam nos líquidos biológicos e tecidos dos pacientes afetados por essa doença, tais como o ácido cis-5-tetradecenóico (Cis-5), que é o marcador para o diagnóstico, e o ácido mirístico (Myr), além de seus derivados de carnitina (VOCKLEY, JERRY et al., 2014).

1.3.1.1 Diagnóstico

O diagnóstico através da triagem neonatal permite o início do tratamento no período pré-sintomático, levando a um melhor prognóstico dos pacientes antes mesmo da manifestação dos sintomas. Desta forma, a triagem neonatal para VLCADD tem efeito benéfico na prevenção dos eventos de hipoglicemia nos

pacientes que possuem alguma atividade residual da enzima. Entretanto, para pacientes que tem atividade residual muito baixa ou ausente, a triagem neonatal não previne a hipoglicemia ou as complicações cardíacas, além de não ser possível estabelecer ainda os efeitos benéficos da triagem neonatal nas complicações relacionadas com a miopatia (BLEEKER et al., 2019).

O diagnóstico dessa doença é feito pelo aumento nas concentrações plasmáticas de acilcarnitinas insaturadas de cadeia longa (principalmente C14:1 e C14:2), podendo ser confirmado por teste enzimático ou molecular. A investigação diagnóstica inclui exames de rotina como dosagem de glicose, lactato, amônia, creatina cinase e eletrólitos, além de análise de ácidos orgânicos na urina que mostra aumento da excreção de ácidos dicarboxílicos. O perfil de acilcarnitinas é crítico para o diagnóstico, especialmente se for feito durante uma crise metabólica aguda e corresponde a um aumento predominante dos níveis de cis-5-tetradecenoilcarnitina (C14:1) (Figura 3). Durante as crises agudas também ocorre o aumento plasmático característico de outras acilcarnitinas (C14:0, C14:2, C16:0, C16:1, C18:0, C18:1 e C18:2), mas entre os períodos de descompensação metabólica somente as C14:1 e C14:2 podem estar elevadas (VOCKLEY, JERRY et al., 2014).

Quando há suspeita de VLCADD, o teste molecular é a forma mais correta de confirmar o diagnóstico. As mutações múltiplas no gene *ACADVL* têm sido estudadas *in vitro* e correlações genótipo/fenótipo têm sido detectadas (GOETZMAN et al., 2007; LAFORET et al., 2009; SCHIFF et al., 2013). Quando são encontradas duas mutações conhecidamente patogênicas, o diagnóstico definitivo é determinado. Entretanto, a atividade enzimática em fibroblastos obtidos de biópsia de pele e os estudos *in vitro* usando palmitato deuterado ou tritiado

podem ajudar no diagnóstico e avaliar o curso clínico (prognóstico) da doença (VIANEY-SABAN et al., 1998).

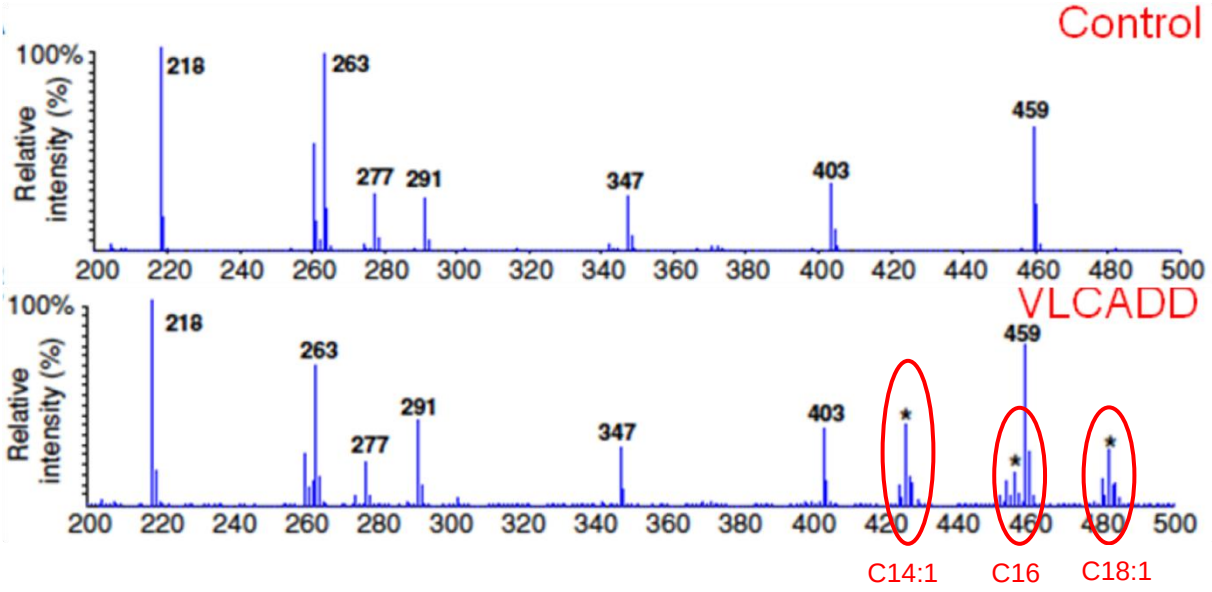

Figura 3. Perfis de acilcarnitinas como seus ésteres butíricos no plasma de um indivíduo normal e de um paciente com VLCADD. Observa-se aumento da tetradecenoilcarnitina (*C14:1; m/z 426), bem como de outras acilcarnitinas de cadeia longa (*C16, m/z 456; *C18:1,m/z 482). O número em cima de cada pico indica a razão m/z para carnitina livre (m/z 218) ou os padrões internos: m/z 263, D3-acetilcarnitina (C2); m/z 277, D3-propionilcarnitina (C3); m/z 291, D3-butirilcarnitina (C4); m/z 347, D3-octanoilcarnitina (C8); m/z 403, D3-dodecanoilcarnitina (C12); m/z 459, D3-palmitoilcarnitina (C16). Adaptado de VOCKLEY, JERRY et al. (2014) e SMITH; MATERN (2010).

1.3.1.2 Tratamento

O objetivo do tratamento em todas as DGOAG é minimizar a oxidação de ácidos graxos evitando jejum e provendo calorias de outras fontes, principalmente durante situações de estresse metabólico (VOCKLEY, JERRY et al., 2014).

No caso da VLCADD, o tratamento consiste na substituição de triglicerídios de cadeia longa da dieta por triglicerídios de cadeia média (MCT) que podem ser totalmente oxidados na β-oxidação mitocondrial. A eficácia clínica do MCT é constatada pela prevenção ou atenuação da cardiomiopatia e dos sintomas musculares que os pacientes apresentam (TUCCI et al., 2013). Além disso, o uso de triglicerídios de cadeia ímpar como a trieptanoína e a suplementação com

bezafibrato (estimulador de função mitocondrial) foram demonstrados ser eficientes ao melhorar a miopatia e a rabdomiólise (BONNEFONT et al., 2010; ROE, C. R. et al., 2002). Recentemente, um ensaio clínico duplo cego randomizado usando trieptanoína *versus* trioctanoína (MCT regular) em DGOAG de cadeia longa concluiu que a triheptanoína melhorou significantemente a fração de ejeção do ventrículo esquerdo, além de reduzir a massa em repouso do ventrículo esquerdo e a frequência cardíaca durante exercício comparado com a trioctanoína (GILLINGHAM et al., 2017). Também foi visto que a trieptanoína diminuiu a duração e taxa de hospitalização e a frequência da hipoglicemia (VOCKLEY, J. et al., 2015), além de melhorar a resistência e tolerância ao exercício e a qualidade de vida dos pacientes (VOCKLEY, J. et al., 2017). Em um estudo de fase 2, o tratamento com trieptanoína por 78 semanas reduziu a frequência de eventos clínicos graves, aumentando a tolerância do exercício e a qualidade de vida. Como a maioria dos pacientes estava utilizando previamente o tratamento com MCT ao tratamento com trieptanoína, as mudanças significativas nos eventos clínicos sugerem que esta última droga significativamente melhora o prognóstico dos pacientes afetados (VOCKLEY, J. et al., 2018).

Já a suplementação com L-carnitina para DGOAG tem sido utilizada para corrigir a deficiência dessa substância e eliminar as acilcarnitinas tóxicas (WINTER, 2003). Entretanto, a utilização de L-carnitina é controversa por falta de comprovação científica (SPIEKERKOETTER et al., 2010) e devido a resultados obtidos em camundongos nocaute com VLCADD mostrando um risco elevado de arritmia causada pelo acúmulo de acilcarnitinas de cadeia longa (SPIEKERKOETTER; LINDNER; SANTER; GROTZKE; BAUMGARTNER; BOEHLES; DAS; HAASE; HENNERMANN; KARALL; KLERK; et al., 2009). Alguns pacientes apresentaram

crises recorrentes de rabdomiólise somente quando estavam fazendo suplementação de L-carnitina (WATANABE et al., 2018).

1.3.1.3 Fisiopatologia

A principal hipótese da fisiopatologia dos defeitos de β-oxidação é atribuída à deficiência de energia devido ao bloqueio da oxidação de ácidos graxos e à baixa disponibilidade de glicose e corpos cetônicos, especialmente em situações de catabolismo acelerado (OLPIN, 2013).

Além disso, tem sido proposta a toxicidade das acilcarnitinas de cadeia longa na patogênese na VLCADD (SPIEKERKOETTER; WOOD, 2010; TUCCI et al., 2013). Também tem sido postulado, mas ainda não demonstrado, que os ácidos graxos de cadeia longa que se acumulam nessa doença possam ser tóxicos para o coração, fígado e músculo esquelético, explicando os sintomas que os pacientes apresentam, principalmente em situações de descompensação metabólica nas quais as concentrações desses ácidos graxos aumentam muito.

Um estudo baseado em ressonância magnética nuclear (RMN) mostrou alterações morfológicas no músculo esquelético de pacientes com VLCADD, bem como aumento dos níveis de creatina fosfocinase e ácido lático associado aos episódios de rabdomiólise, sugerindo um distúrbio na homeostase energética mitocondrial nesse tecido. Também foi observado em imagens de RMN de músculo esquelético de pacientes afetados um aumento do conteúdo de lipídios devido à lise celular ou secundário a processo inflamatório, indicando toxicidade desses compostos acumulados (DIEKMAN et al., 2014).

Para melhor estudar essa doença foi desenvolvido um modelo nocaute em camundongos, nos quais se manifesta o fenótipo semelhante ao que acomete os

seres humanos afetados. Esses animais apresentaram distúrbio bioenergético mitocondrial (EXIL et al., 2006), refletido pela diminuição do razão fosfocreatina/ adenosina trifosfato (ATP) (TUCCI et al., 2014) e dos níveis de ATP (XIONG et al., 2014) que foram associados à disfunção cardíaca. Exil e colaboradores (2003) mostraram em diferentes tecidos de camundongos nocaute para VLCAD proliferação mitocondrial acelerada, que presumivelmente foi atribuída à β-oxidação comprometida associada à deficiência de energia. Além disso, também se observou através de RMN que a função cardíaca desses animais estava comprometida com um volume sistólico significativamente menor em comparação com camundongos do tipo selvagem (TER VELD et al., 2007). Também foi demonstrada através de RMN uma diminuição da fração de ejeção acompanhada por aumento de triglicerídeos e ceramidas no miocárdio de animais em jejum, sugerindo que o acúmulo dos compostos lipotóxicos possam contribuir para o desequilíbrio metabólico detectado durante jejum (BAKERMANS et al., 2013). Ademais, SPIEKERKOETTER et al. (2006) mostraram uma redução na taxa de gliconeogênese devido à diminuição dos níveis de di-hidroxiacetona fosfato e glicerol-3-fosfato, sugerindo prejuízo na disponibilidade de precursores de glicose a partir de glicerol.

Tendo em vista que a patogênese do dano tecidual múltiplo encontrado na VLCADD é pouco conhecida e a função mitocondrial, principalmente a homeostase bioenergética, é crítica para o funcionamento normal dos tecidos e órgãos ricos em mitocôndrias, descrevemos a seguir alguns aspectos importantes desse sistema celular que foram objetos de avaliação no presente trabalho.

1.4 MITOCÔNDRIA E FOSFORILAÇÃO OXIDATIVA

A mitocôndria é a organela responsável pela regeneração de adenosina difosfato (ADP) a ATP através da fosforilação oxidativa. Além disso, é nessa organela que há a maior produção de espécies reativas de oxigênio como o superóxido e o peróxido de hidrogênio, e também de defesas antioxidantes (CADENAS; DAVIES, 2000; KANDOLA; BOWMAN; BIRCH-MACHIN, 2015). É também responsável pela homeostase do cálcio e participa dos processos de morte celular (FINKEL et al., 2015; ORRENIUS; GOGVADZE; ZHIVOTOVSKY, 2015). É envolta por duas membranas, uma externa permeável a pequenas moléculas e íons, e uma interna impermeável à maioria das moléculas, incluindo ATP, ADP, fosfato inorgânico, piruvato, H^+, NADH, nicotinamida adenina dinucleotídeo (NAD^+), entre outras (NELSON; COX, 2017).

Os glicídeos, aminoácidos e ácidos graxos podem ser oxidados no CAC, entrando sob a forma de acetil-CoA ou outros intermediários do CAC e então oxidados completamente a CO_2, fornecendo as coenzimas reduzidas NADH e $FADH_2$ que irão abastecer com elétrons a cadeia respiratória mitocondrial (NICHOLLS; FERGUSON, 2013).

A cadeia respiratória é composta por várias associações proteicas (complexos enzimáticos I, II, III, IV e V), bem como pelo citocromo *c* e por uma coenzima lipossolúvel (ubiquinona ou Coenzima Q10) (DI DONATO, 2000). NADH e $FADH_2$ fornecem elétrons para os complexos I e II, respectivamente, sendo estas as vias convencionais de entrada de elétrons. 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 CAC. 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; JENKS, 1992). O fluxo de elétrons através dos complexos da cadeia respiratória é acompanhado pelo bombeamento de prótons da matriz mitocondrial para o espaço intermembrana, através dos complexos I, III e IV, gerando um gradiente eletroquímico que pode ser utilizado pelo complexo V (ATP sintase) para a síntese de ATP. Dessa forma, o oxigênio permite a respiração mitocondrial (fosforilação oxidativa - OXPHOS) através da oxidação de substratos energéticos acoplado ao processo de fosforilação do ADP, ou seja, quando a energia do gradiente eletroquímico é dissipada pelo fluxo de prótons de volta para a matriz mitocondrial, a energia liberada é utilizada pela ATP sintase para sintetizar moléculas de ATP, permitir o movimento de cálcio e outras moléculas para dentro e fora da mitocôndria (NELSON; COX, 2017; NICHOLLS; FERGUSON, 2013).

A respiração mitocondrial pode ser estimada através da medida do consumo de O_2, sendo um excelente sensor da atividade da cadeia respiratória mitocondrial, uma vez que os processos de transferência de elétrons, bombeamento de prótons, formação do gradiente eletroquímico e síntese de ATP são acoplados e estão interligados ao consumo de O_2. Experimentalmente, pode-se dividir a respiração mitocondrial em 5 estados respiratórios, sendo que os estados 3 e 4 são os mais comumente utilizados. O estado 3 representa o consumo de oxigênio quando as mitocôndrias, em um meio contendo substrato oxidável capaz de

estimular o fornecimento de elétrons para a cadeia respiratória, são expostas a ADP, estimulando o consumo de O_2 e produzindo ATP (estado fosforilante). O estado 4 geralmente é estimulado por oligomicina, um inibidor da ATP sintase, refletindo o consumo de O_2 necessário para a formação e manutenção do gradiente eletroquímico, reduzindo a taxa da respiração (estado não-fosforilante). Ainda, é possível utilizar compostos desacopladores da fosforilação oxidativa, como o cianeto de carbonila de meta-clorofenil-hidrazona (CCCP), os quais provocam um vazamento de prótons do espaço intermembrana para a matriz mitocondrial, estimulando a atividade da cadeia respiratória mitocondrial e o consumo de O_2 sem produção de ATP (estado desacoplado) (NICHOLLS; FERGUSON, 2013).

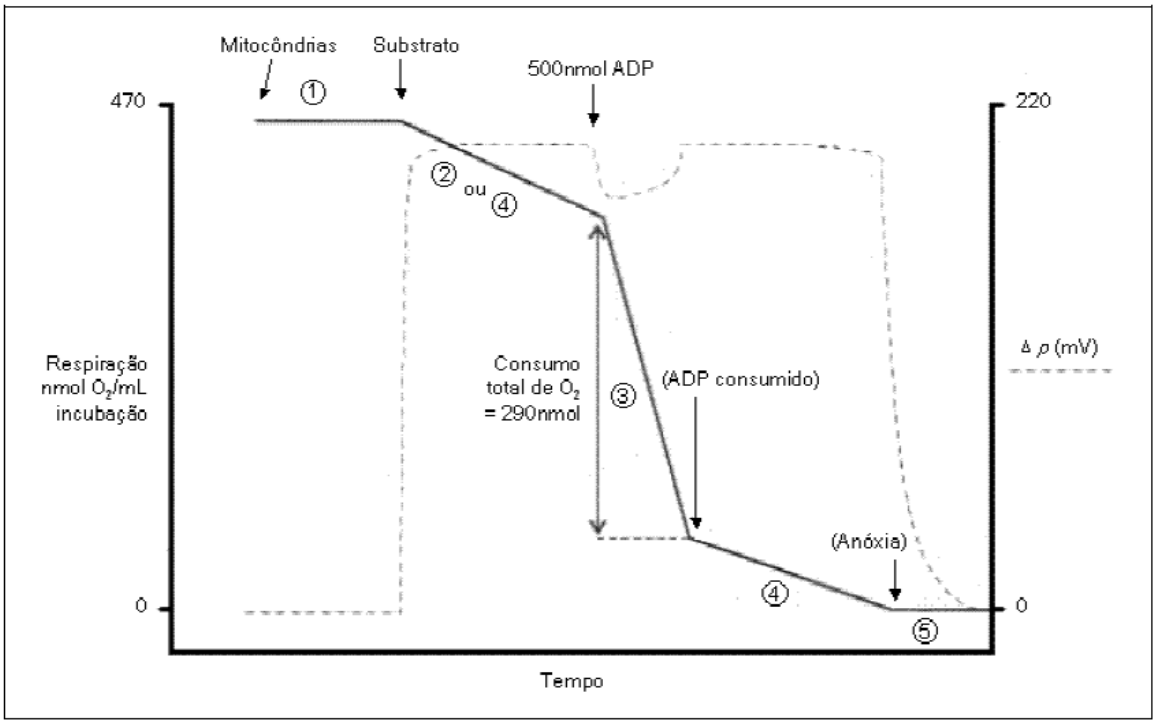

Figura 4. Estados da respiração mitocondrial. As mitocôndrias são adicionadas a uma cuba com um eletrodo de oxigênio (estado 1), seguida da adição de substrato (estado 2) (há fosfato inorgânico disponível). A respiração é lenta pois o circuito de prótons não está completo com a reentrada de H^+ pela ATP sintase. Existe uma respiração devido ao vazamento de prótons pela membrana. É adicionado ADP, permitindo a síntese de ATP pela ATP sintase acoplada à reentrada de prótons (estado 3). Quando todo o ADP adicionado é convertido a ATP a respiração fica lenta novamente (estado 4) até que finalmente o oxigênio da cuba termina levando à anóxia (estado 5) (Adaptado de NICHOLLS; FERGUSON, 2002)

É possível medir experimentalmente, além da respiração mitocondrial, o potencial de membrana (ΔΨm) através da fluorescência do reagente catiônico safranina O, o inchamento por meio da turbidez e da luz espalhada da amostra, a produção de peróxido de hidrogênio pela reação acoplada onde a peroxidase oxida o reagente ampliflu red formando resorufina que emite fluorescência ao mesmo tempo em que transforma o H_2O_2 em O_2. A capacidade de retenção de Ca^{2+} é medida pela fluorescência do detector de cálcio *calcium green* e o conteúdo de nicotinamida adenina dinucleotídeo (fosfato) reduzida (NAD(P)H) mitocondrial é medido pela autofluorescência desses compostos. (MACIEL et al., 2004; SAITO; CASTILHO, 2010).

1.5 PAPEL DA MITOCÔNDRIA NA HOMEOSTASE DO Ca^{2+}

Uma função crucial desempenhada por mitocôndrias é manter a homeostase celular de Ca^{2+}, já que ele é fundamental para sinalizar e controlar uma série de eventos intracelulares. Essa organela tem a capacidade, quando íntegra e funcional, 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 (FIGUEIRA et al., 2013; RIZZUTO et al., 2012). A proteína transportadora mitocondrial responsável pela captação do Ca^{2+} foi recentemente elucidada, sendo um sistema uniporte de captação de Ca^{2+} (MCU) (figura 5) (BAUGHMAN et al., 2011; DE STEFANI et al., 2011; MARCHI; PINTON, 2014; PAN et al., 2013; PENDIN; GREOTTI; POZZAN, 2014). Por outro lado, a liberação de Ca^{2+} para a matriz é realizada pelos trocadores Na^+/Ca^{2+} (mNCX) e H^+/Ca^{2+} (mHCX) (BERNARDI, P.; VON STOCKUM, 2012; RIZZUTO et al., 2012).

Quando a célula está com sobrecarga citosólica de Ca^{2+}, que pode ser muito prejudicial para o funcionamento celular, a capacidade da mitocôndria de captar e reter Ca^{2+} contribui para o tamponamento do excesso citosólico desse cátion (FIGUEIRA et al., 2013; RIZZUTO et al., 2012). Por outro lado, uma elevada captação mitocondrial de Ca^{2+}, além da sua capacidade de retenção, pode levar a uma condição conhecida como transição de permeabilidade, resultado da abertura de um poro na membrana mitocondrial interna (ADAM-VIZI; STARKOV, 2010; STARKOV, 2010; ZORATTI; SZABO, 1995). O poro de transição de permeabilidade (PTP) mitocondrial é formado por proteínas com identidades ainda pouco conhecidas (STARKOV, 2010), apesar de já ser reconhecido o papel da ciclofilina D (Cyp D) como um componente chave para a abertura do PTP (BAINES et al., 2005; BASSO et al., 2005; BERNARDI, PAOLO, 2013; TANVEER et al., 1996).

A permanente abertura do PTP resulta em consequências funestas para a célula, tais como a liberação de Ca^{2+} para o citosol, inchamento mitocondrial, liberação de fatores apoptogênicos intramitocondriais como o citocromo *c* (LIU et al., 1996), despolarização mitocondrial, perda de íons e metabólitos (Ca^{2+}, Mg^{2+}, glutationa reduzida (GSH), NADH e NADPH), comprometimento da síntese de ATP e morte celular (apoptose e necrose) (BERNARDI, P.; VON STOCKUM, 2012; CROMPTON et al., 1999; RASOLA; BERNARDI, 2011; RIZZUTO et al., 2012). Muitos trabalhos *in vitro* e *in vivo* têm associado a indução do PTP com uma disfunção mitocondrial na presença de Ca^{2+}, demonstrando alteração nos parâmetros da homeostase bioenergética mitocondrial (MACIEL et al., 2004; MIRANDOLA et al., 2010; TONIN et al., 2014). Além disso, o ataque oxidativo por espécies reativas de oxigênio está descrito como um importante mecanismo potencializador da abertura do PTP (ADAM-VIZI; STARKOV, 2010;

KOWALTOWSKI; CASTILHO; VERCESI, 2001). 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 (HOEK; RYDSTROM, 1988; LEHNINGER; VERCESI; BABABUNMI, 1978; ZAGO; CASTILHO; VERCESI, 2000).

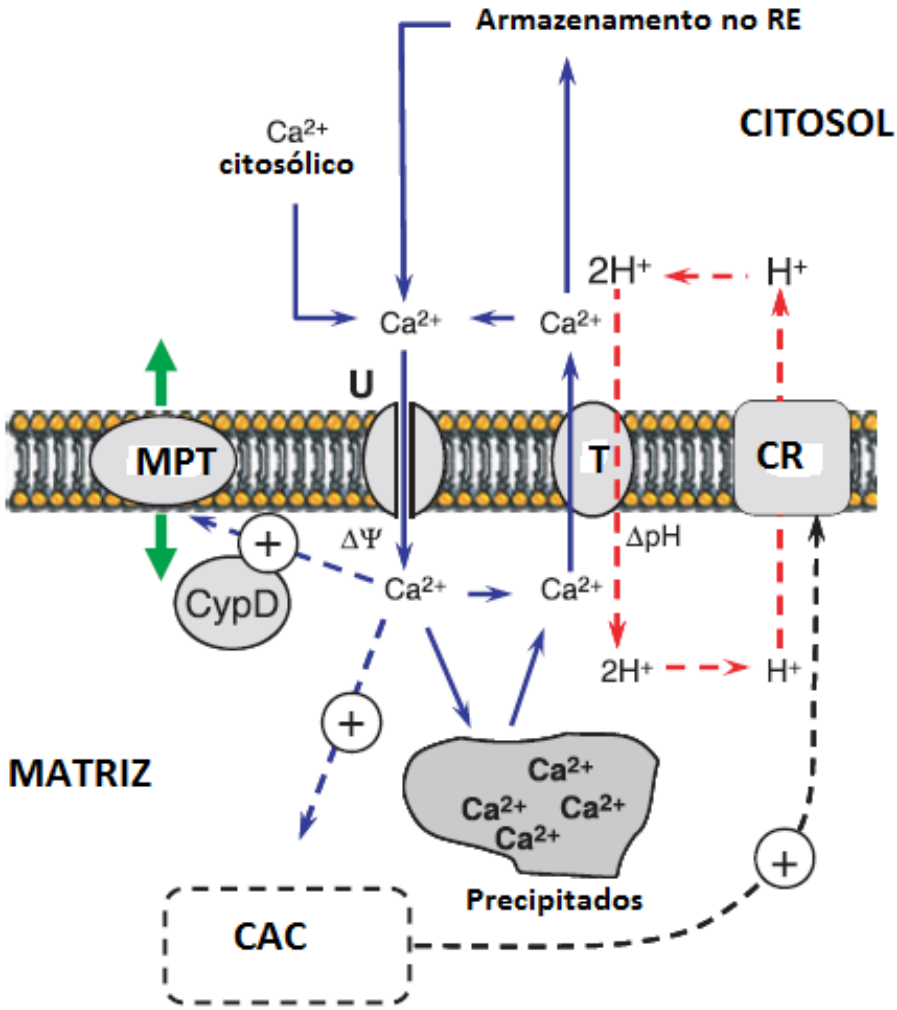

Figura 5. Modelo padrão da homeostase mitocondrial de Ca^{2+}. As mitocôndrias acumulam Ca^{2+} exógeno por meio do transportador eletrogênico (uniporte de Ca^{2+}, U) que facilita o transporte de Ca^{2+} através da membrana mitocondrial interna para a matriz. O transporte é acoplado ao acúmulo simultâneo 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 um certo limiar, desencadeia-se a abertura do PTP, a qual também é modulada pela proteína localizada na matriz ciclofilina D (Cyp D). T: trocador; CR: cadeia respiratória (Adaptado de Starkov (2010).

2. OBJETIVO GERAL

Investigar os efeitos dos ácidos cis-5-tetradecenóico (Cis-5) e mirístico (Myr), principais ácidos graxos acumulados na VLCADD, sobre importantes funções mitocondriais no coração, fígado e músculo esquelético de ratos jovens, bem como em cardiomiócitos e hepatócitos cultivados, com a finalidade de esclarecer a patogênese do dano tecidual que acomete os pacientes afetados pela VLCADD, causando cardiomiopatia, insuficiência hepática e sintomas musculares esqueléticos.

2.1 OBJETIVOS ESPECÍFICOS

- Investigar os efeitos do Cis-5 e Myr sobre a respiração celular (parâmetros respiratórios: estado 3, estado 4, estado desacoplado e razão de controle respiratório - RCR) e a produção de ATP em preparações mitocondriais de coração, fígado e músculo esquelético de ratos adolescentes;
- Investigar os efeitos do Cis-5 e Myr sobre a respiração mitocondrial (parâmetros respiratórios: estado 3, estado 4 e estado desacoplado) em fibras permeabilizadas de coração e de músculo esquelético de ratos adolescentes, bem como em cardiomiócitos (linhagem H9C2) e hepatócitos (linhagem HepG2) permeabilizados;
- Investigar os efeitos do Cis-5 e Myr sobre o potencial de membrana mitocondrial, conteúdo de NAD(P)H e capacidade de retenção de cálcio em preparações mitocondriais provenientes de coração, fígado e músculo esquelético de ratos adolescentes, bem como sobre o inchamento mitocondrial e a liberação de citocromo *c* por mitocôndrias de fígado, na presença de cálcio;

- Investigar os efeitos do Cis-5 e Myr sobre as atividades dos complexos da cadeia transportadora de elétrons e das enzimas glutamato desidrogenase (GDH), malato desidrogenase (MDH), sucinato desidrogenase (SDH), α-cetoglutarato desidrogenase (α-KGDH), isocitrato desidrogenase (IDH), citrato sintase (CS) e aspartato aminotransferase (AST) em preparações mitocondriais de coração, fígado e músculo esquelético de ratos adolescentes;
- Investigar os efeitos do Cis-5 e Myr sobre a fluidez de membrana em preparações mitocondriais de coração e fígado de ratos adolescentes.

PARTE II: Artigos Científicos

CAPÍTULO I

Metabolite accumulation in VLCAD deficiency markedly disrupts mitochondrial bioenergetics and Ca^{2+} homeostasis in the heart

Cristiane Cecatto, Alexandre Umpierrez Amaral, Janaína Camacho da Silva, Alessandro Wajner, Mariana de Oliveira Vargas Schimit, Lucas Henrique Rodrigues da Silva, Simone Magagnin Wajner, Ângela Zanatta, Roger Frigério Castilho e Moacir Wajner

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Metabolite accumulation in VLCAD deficiency markedly disrupts mitochondrial bioenergetics and Ca^{2+} homeostasis in the heart

Cristiane Cecatto[1], Alexandre Umpierrez Amaral[1,2], Janaína Camacho da Silva[1], Alessandro Wajner[1], Mariana de Oliveira Vargas Schimit[1], Lucas Henrique Rodrigues da Silva[1], Simone Magagnin Wajner[3], Ângela Zanatta[1], Roger Frigério Castilho[4] and Moacir Wajner[1,5,6]

1 Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
2 Departamento de Ciências Biológicas, Universidade Regional Integrada do Alto Uruguai e das Missões, Erechim, Brazil
3 Departamento de Medicina Interna, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
4 Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Brazil
5 Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
6 Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Brazil

Keywords
cis-5-tetradecenoic acid; mitochondrial Ca^{2+} homeostasis; mitochondrial energy homeostasis; myristic acid; very long-chain acyl-CoA dehydrogenase

Correspondence
M. Wajner, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul, Rua Ramiro Barcelos, 2600 – Anexo, CEP 90035-003, Porto Alegre, RS, Brazil
Fax: +55 51 3308 5540
Tel: +55 51 3308 5571
E-mail: mwajner@ufrgs.br

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We studied the effects of the major long-chain fatty acids accumulating in very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, namely *cis*-5-tetradecenoic acid (Cis-5) and myristic acid (Myr), on important mitochondrial functions in isolated mitochondria from cardiac fibers and cardiomyocytes of juvenile rats. Cis-5 and Myr at pathological concentrations markedly reduced mitochondrial membrane potential ($\Delta\Psi_m$), matrix NAD(P)H pool, Ca^{2+} retention capacity, ADP- (state 3) and carbonyl cyanide 3-chlorophenyl hydrazine-stimulated (uncoupled) respiration, and ATP generation. By contrast, these fatty acids increased resting (state 4) respiration (uncoupling effect) with the involvement of the adenine nucleotide translocator because carboxyatractyloside significantly attenuated the increased state 4 respiration provoked by Cis-5 and Myr. Furthermore, the classical inhibitors of mitochondrial permeability transition (MPT) pore cyclosporin A plus ADP, as well as the Ca^{2+} uptake blocker ruthenium red, fully prevented the Cis-5- and Myr-induced decrease in $\Delta\Psi_m$ in Ca^{2+}-loaded mitochondria, suggesting, respectively, the induction of MPT pore opening and the contribution of Ca^{2+} toward these effects. The findings of the present study indicate that the major long-chain fatty acids that accumulate in VLCAD deficiency disrupt mitochondrial bioenergetics and Ca^{2+} homeostasis, acting as uncouplers and metabolic inhibitors of oxidative phosphorylation, as well as inducers of MPT pore opening, in the heart at pathological relevant concentrations. It is therefore presumed that a disturbance of bioenergetics and Ca^{2+} homeostasis may contribute to the cardiac manifestations observed in VLCAD deficiency.

Abbreviations
ANOVA, one-way analysis of variance; ANT, adenine nucleotide translocator; CI, complex I; CII, complex II; CAT, carboxyatractyloside; CCCP, carbonyl cyanide 3-chlorophenyl hydrazine; Cis-5, *cis*-5-tetradecenoic acid; CsA, cyclosporin A; DPC, diethyl pyrocarbonate; EtOH, ethanol; FAU, fluorescence arbitrary units; GDH, glutamate dehydrogenase; GSH, reduced glutathione; LCFA, long-chain fatty acids; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; Mal, malonate; MDA, malondialdehyde; MDH, malate dehydrogenase; MPT, mitochondrial permeability transition; Myr, myristic acid; OXPHOS, oxidative phosphorylation; PMG, pyruvate plus malate plus glutamate; RCR, respiratory control ratio; RR, ruthenium red; SDH, succinate dehydrogenase; SUIT, substrate-uncoupler inhibitor titration; VLCAD, very long-chain acyl-CoA dehydrogenase; $\Delta\Psi_m$, mitochondrial membrane potential.

Introduction

Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency (OMIM 609575) is the most frequent disorder of long-chain fatty acid (LCFA) β-oxidation, with an incidence of 1 : 50 000 to 1 : 100 000 in newborns [1,2]. It is biochemically characterized by the accumulation of LCFA and carnitine derivatives. Diagnosis is performed by tandem mass spectrometry in dried blood by elevation of C14 to C18 acylcarnitines, in which C14:1 (*cis*-5-tetradecenoylcarnitine) is considered as a disease-specific marker [3–5]. Although heterogeneous, the disorder is usually manifested by three clinical forms based on disease severity: (a) a severe, early-onset presentation with predominating cardiomyopathy with or without pericardial effusion leading to high mortality rates; (b) an infancy-onset hepatic phenotype with recurrent hypoketotic hypoglycemia; and (c) a late-onset, myopathic form with muscle weakness, myalgia, episodic myoglobinuria and rhabdomyolysis. The major cardiac manifestations include arrhythmia, conduction abnormalities, cardiomyopathy with or without pericardial effusion, and sudden death mainly induced by fasting, exercise, illness and fever [3,6–14]. VLCAD deficiency was included in newborn screening programs because of the life-threatening symptoms that can be prevented or attenuated by dietary regimens in a considerable number of patients [2,15].

Treatment for VLCAD deficiency is based on frequent meals and the avoidance of long fasting to prevent catabolism, as well as medium-chain triglyceride supplementation to provide the energy required by tissues that mainly consume fatty acids [2,16]. More recently, supplementation of triheptanoin to patients with fatty acid oxidation defects manifesting as cardiomyopathy and persistent myopathy has been proposed, with promising results being obtained, because this compound leads to synthesis of the ketones β-hydroxypentanoate and β-ketopentanoate, which, together with propionyl-CoA, serve as anaplerotic substrates [17,18], although the efficacy of this diet is still under investigation. The natural polyphenol resveratrol with antioxidant and anti-inflammatory properties was recently shown to be beneficial in human fibroblasts of patients with LCFA disorders [19]. Finally, bezafibrate was demonstrated to activate the peroxisome proliferator-activated receptor, leading to higher residual dehydrogenase activity and therefore restoring fatty acid oxidation capacities in fibroblasts of VLCAD-deficient patients [20].

With regard to the pathophysiology of mitochondrial LCFA β-oxidation disorders, the main current hypothesis for the development of the severe clinical symptoms is attributed to energy deficiency as a result of the blockage of fatty acid oxidation and a low availability of glucose (hypoglycemia) and ketone bodies, especially during periods of fasting and illness [21]. However, more recently, the potential toxicity of very long-chain acylcarnitines was also proposed to play a role in the pathogenesis of this disease [16,22]. Therefore, it could be also considered that LCFA accumulating in this disorder may be cardiotoxic and important with respect to explaining the cardiomyopathy that affects patients, particularly during crises of metabolic decompensation in which the concentrations of these fatty acids increase dramatically. Noteworthy, morphological mitochondrial alterations in skeletal muscle detected by magnetic resonance imaging, as well as increased blood levels of creatine phosphokinase and lactic acid associated with episodic rhabdomyolysis, were found in VLCAD-deficient patients [23,24], suggesting a disturbance of mitochondrial energy homeostasis, although, to the best of our knowledge, ATP production was not measured in these patients. Similarly, there is evidence of bioenergetics disruption in the hearts of VLCAD-deficient mice, including a decrease in the phosphoreatine/ATP ratio [25], as well as the ATP pool [26], both associated with cardiac dysfunction in mice. In addition, we previously demonstrated that long-chain hydroxylated fatty acids accumulating in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency strongly disturb mitochondrial functions in various tissues [27–29]. Therefore, in the present study, we investigated the effects of *cis*-5-tetradecenoic (Cis-5; C14:1) and myristic (Myr; C14) acids at physiological ($\leq$ 10 μM) and pathological concentrations (30–100 μM) [30] on important endpoints of mitochondrial function, including membrane potential ($\Delta\Psi_m$), matrix NAD(P)H content, Ca^{2+} retention capacity and ATP production in Ca^{2+}-unloaded and loaded mitochondrial preparations from the hearts of juvenile rats. The effects of these fatty acids on oxidative phosphorylation (OXPHOS) were also investigated by evaluating, ADP- (state 3), carbonyl cyanide 3-chlorophenyl hydrazine (CCCP)-(uncoupled) stimulated and resting (state 4) respiration, as well as some respiratory chain complexes and citric acid cycle enzyme activities, in mitochondria isolated from the heart. Some of these parameters were also measured in heart fibers and cardiomyocytes that reflect integrated cell systems and better mimic the cell milieu *in vivo*. Finally, we investigated whether Cis-5 and Myr could alter some important parameters of redox homeostasis.

Results

Myr and Cis-5 decrease $\Delta\Psi_m$ in heart mitochondria

We first assessed the effects of Myr (5–100 μM) and Cis-5 (1–100 μM) on $\Delta\Psi_m$ in state 4 (resting) respiring heart mitochondria before and after Ca^{2+} addition using glutamate plus malate as substrates. Myr and Cis-5 significantly decreased $\Delta\Psi_m$ in the absence of Ca^{2+} at 30 μM and higher concentrations in a dose-dependent manner, indicating an uncoupling effect. It was also observed that this effect was enhanced when mitochondrion was challenged by Ca^{2+} (Mir: $F_{5,18}$ = 51.410, $P < 0.001$; Cis-5: $F_{5,18}$ = 151.912, $P < 0.001$) (Fig. 1A,B). Interestingly, at physiological concentrations, both fatty acids were unable to change $\Delta\Psi_m$, highlighting their roles at the pathological concentrations found in the affected patients, particularly during metabolic decompensation. Furthermore, Myr- and Cis-5-induced mitochondrial depolarization in the presence of Ca^{2+} was prevented by ruthenium red (RR), which blocks mitochondrial Ca^{2+} uptake, as well as by the classical inhibitors of mitochondrial permeability transition (MPT) cyclosporin A (CsA) plus ADP [Mir: $F_{5,23}$ = 28.212, $P < 0.001$); Cis-5: $F_{3,17}$ = 84.468, P < 0.001) (Fig. 1C,D), indicating that these fatty acids elicit MPT in a mechanism dependent on the presence of Ca^{2+}. We also confirmed that the adenine nucleotide translocator (ANT) inhibitor carboxyatractyloside (CAT) totally prevented the Myr-induced uncoupling effect (decrease in $\Delta\Psi_m$ in a medium devoid of Ca^{2+}), in contrast to the potent reducing agent dithiothreitol that was unable to change $\Delta\Psi_m$ dissipation caused by Myr (Fig. 1C).

Myr and Cis-5 reduce mitochondrial matrix NAD(P)H content in heart mitochondria

Next, we evaluated the effects of Myr and Cis-5 on matrix NAD(P)H content in glutamate plus malate-supported heart mitochondria. We observed that both fatty acids induced a strong decrease in NAD(P)H levels in the absence of Ca^{2+} (Fig. 2). Furthermore, NAD(P)H levels were fully depleted after Ca^{2+} addition, suggesting that the reduced equivalents were oxidized or lost from the mitochondrial matrix.

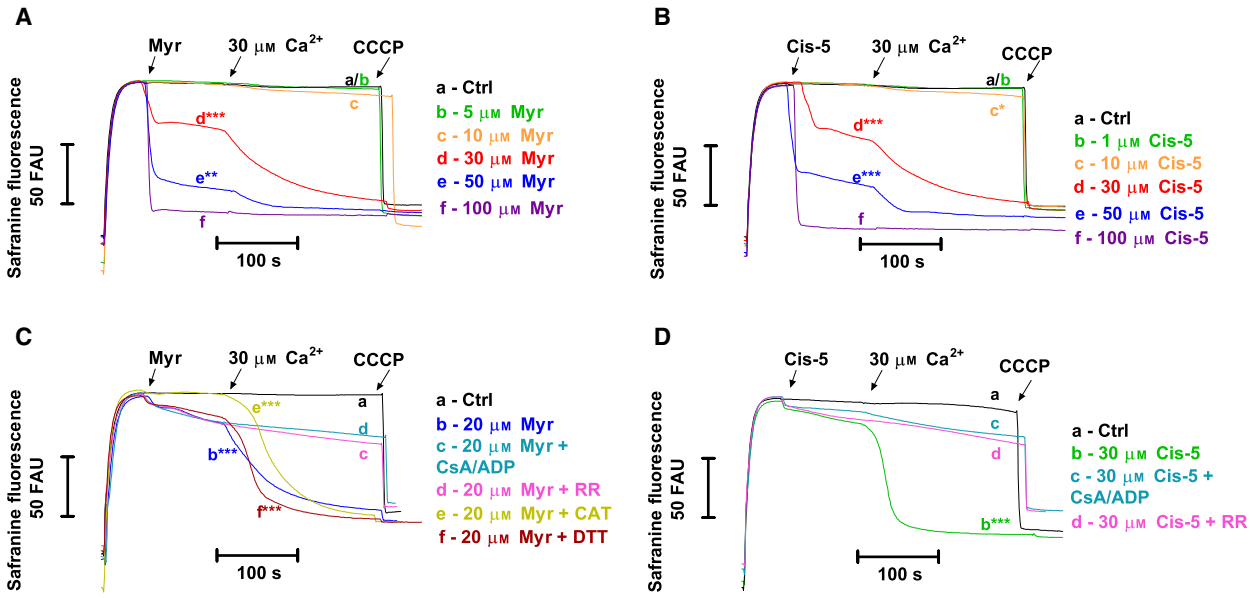

Fig. 1. Effects of Myr and Cis-5 acids on mitochondrial membrane potential in heart mitochondria. All experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein·mL^{-1}) supported by glutamate plus malate (2.5 mM each). (A) Myr (5–100 μM, lines b–f) or (B) Cis-5 (1–100 μM, lines b–f) were added 50 s after the beginning of the assay. (C) Myr (20 μM, lines b–f). (D) Cis-5 (30 μM, lines b–d). CsA (1 μM) plus ADP (300 μM) (line c), RR (1 μM, line d), CAT (1 μM, line e) or dithiothreitol (5 mM, line f) were added at the beginning of the assay. All panels refer to mitochondrial preparations before and after the addition of 30 μM Ca^{2+}, as indicated. Controls (lines a) were performed in the absence of fatty acids. CCCP was added at the end of the assays. Traces are representative of three independent experiments (N) and are expressed as FAU. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ compared to controls (Duncan's multiple range test).

Myr and Cis-5 decrease Ca^{2+} retention capacity in heart and skeletal muscle mitochondria

Because 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 Myr (5–100 μM) and Cis-5 (1–100 μM) using glutamate plus malate as substrates. It can be seen in Fig. 3A,B that both fatty acids markedly reduced the mitochondrial Ca^{2+} retention capacity at concentrations of 30 μM and higher, whereas, at physiological concentrations, there was no effect at all. The results also suggest that the effect of Myr was stronger than that of Cis-5 (Fig. 3C). This was better observed when testing simultaneously the effects of 30 μM Myr and Cis-5 on Ca^{2+} retention capacity using a single Ca^{2+} addition (Fig. 3D). Finally, we tested whether Myr and Cis-5 could change this parameter in skeletal muscle. Both fatty acids were observed to decrease Ca^{2+} retention capacity at concentrations of 30 μM and higher with no change at physiological concentrations (Fig. 3E).

Myr and Cis-5 change oxygen consumption in heart mitochondria

Next, we studied the effects of Myr and Cis-5 on heart mitochondrial respiratory parameters measured by oxygen consumption using glutamate plus malate or succinate as substrates. Myr and Cis-5 (10–30 μM) significantly increased resting (state 4) ($F_{6,30}$ = 32.872, P < 0.001) (Fig. 4B), as well as decreased ADP-stimulated (state 3) ($F_{(6,30}$ = 91.493, P < 0.001) (Fig. 4A), CCCP-stimulated (uncoupled) respiration ($F_{6,30}$ = 27.280, P < 0.001) (Fig. 4C) and respiratory control ratio (RCR) ($F_{6,30}$ = 359.787, P < 0.001) (Fig. 4D), in a dose-dependent manner with glutamate plus malate as substrates.

Disturbance of mitochondrial respiration caused by Myr or Cis-5 was also observed in succinate-supported mitochondria but with less intense effects compared to glutamate plus malate [state 3 (ADP-stimulated): $F_{6,31}$ = 28.782, P < 0.001; state 4 (resting): $F_{6,31}$ = 29.553, P < 0.001; uncoupled (CCCP-stimulated) respiration: $F_{6,31}$ = 14.479, P < 0.001; RCR: $F_{6,31}$ = 303.200, P < 0.001] (Fig. 5A–D). It was also found that Myr-induced decrease in state 3 (ADP-stimulated) respiration supported by succinate was similar to that provoked by the classical succinate dehydrogenase (SDH) inhibitor malonate (Mal). Furthermore, Myr-induced inhibition was partially prevented by alamethicin that permeabilizes mitochondria (Fig. 5E). However, alamethicin was unable to change the metabolic inhibition caused by Mal (Fig. 5E). Taken together, it may be presumed that competition between Myr and succinate for the same mitochondrial membrane transporter is involved in the decrease in ADP-activated respiration by Myr ($F_{5,23}$ = 55.973, P < 0.001) (Fig. 5E).

We also confirmed that Myr-induced increase in resting respiration (uncoupling behavior) was attenuated by the ANT inhibitor CAT ($F_{5,17}$ = 40.850, P < 0.001) (Fig. 6A) but not by the UCP inhibitor GDP ($F_{4,19}$ = 16.943, P < 0.001) (Fig. 6B) or the glutamate/aspartate antiporter inhibitor diethyl pyrocarbonate (DPC) ($F_{5,23}$ = 117.320, P < 0.001) (Fig. 6C), suggesting a role for ANT in this effect.

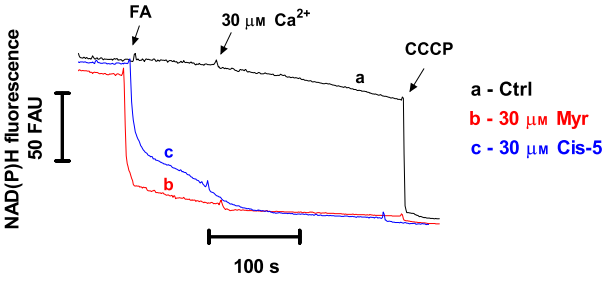

Fig. 2. Effects of Myr and Cis-5 acids on mitochondrial NAD(P)H content in heart mitochondria. All experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein·mL^{-1}) supported by glutamate plus malate (2.5 mM each). Myr or Cis-5 (30 μM, lines b–c) were added 50 s after the beginning of the assay. All traces refer to mitochondrial preparations before and after the addition of 30 μM Ca^{2+}, as indicated. Controls (line a) were performed in the absence of fatty acids. CCCP was added at the end of the assays. Traces are representative of three independent experiments (N) and are expressed as FAU.

Myr and Cis-5 significantly decrease complex I (CI) activity in mitochondrial preparations

Myr and Cis-5 markedly decreased CI activity ($F_{3,20}$ = 64.014, P < 0.001) (Fig. 7A), whereas complex II (CII) activity was not changed by these compounds (Fig. 7B), implying a selective inhibitory effect on CI activity. These results may be partly related to the significant metabolic inhibition provoked by these LCFA on mitochondrial respiration measured by oxygen consumption when glutamate plus malate rather than succinate was used as a mitochondrial respiring substrate. By contrast, Myr and Cis-5 did not alter glutamate dehydrogenase (GDH), SDH and malate dehydrogenase (MDH) activities, indicating that GDH and citric acid cycle inhibition were not involved in the Myr and

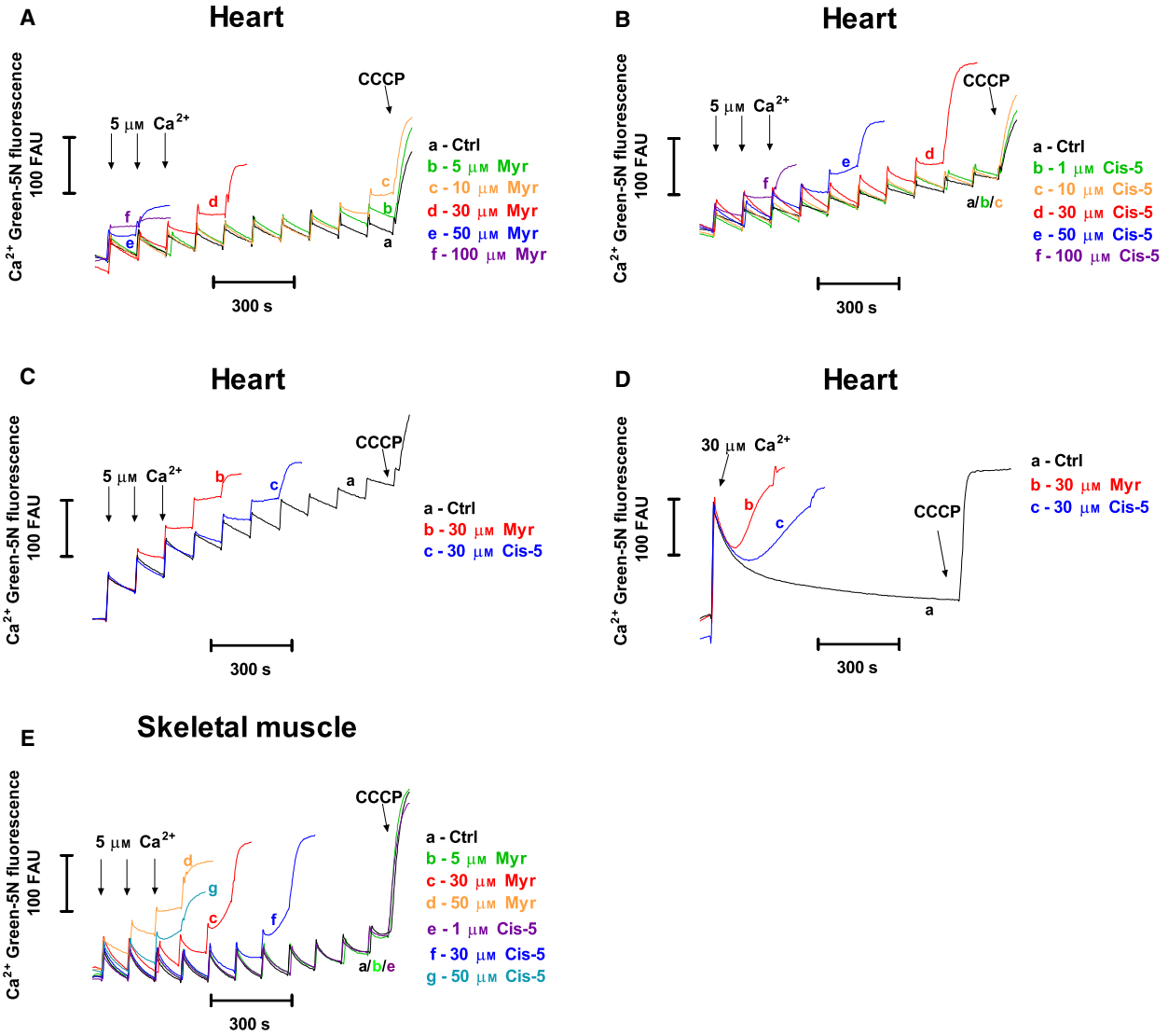

Fig. 3. Effects of Myr and Cis-5 acids on mitochondrial Ca^{2+} retention capacity in Ca^{2+}-loaded heart and skeletal muscle mitochondria. All experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein·mL^{-1}) (Heart: A, B, C and D; skeletal muscle: E) supported by glutamate plus malate (2.5 mM each). The fatty acids were added at the beginning of the assays. (A) Myr (5–100 μM, lines b–f). (B) Cis-5 (1–100 μM, lines b–f). (C, D) Myr or Cis-5 (30 μM, lines b, c). (E) Myr (5–50 μM, lines b–d) or Cis-5 (1–50 μM, lines e–g). (D) Single addition of 30 μM Ca^{2+}. In (A), (B), (C) and (E), mitochondrial preparations were supplemented by successive additions of 5 μM Ca^{2+} every 2 min, as indicated by the arrows. Controls (line a) were performed in the absence of these fatty acids. CCCP was added at the end of the assays. Traces are representative of three independent experiments (N) and are expressed as FAU.

Cis-5-induced decrease in mitochondrial oxygen consumption (Table 1).

Myr and Cis-5 do not change mitochondrial membrane fluidity

Myr and Cis-5 might also interact with mitochondrial membrane lipids, altering fluidity and therefore interfering with some of the parameters examined in the present study, such as the uncoupling effect, induction of MPT and the decrease in CI activity of the respiratory chain. We therefore evaluated the fluidity of mitochondrial membranes by measuring fluorescence anisotropy (r) in the presence of Myr and Cis-5. Triton X-100 was used as a positive control as a result of its strong detergent activity. We observed that both fatty acids did not change this parameter ($F_{3,15} = 21.675$, $P < 0.001$) (Fig. 8), implying that

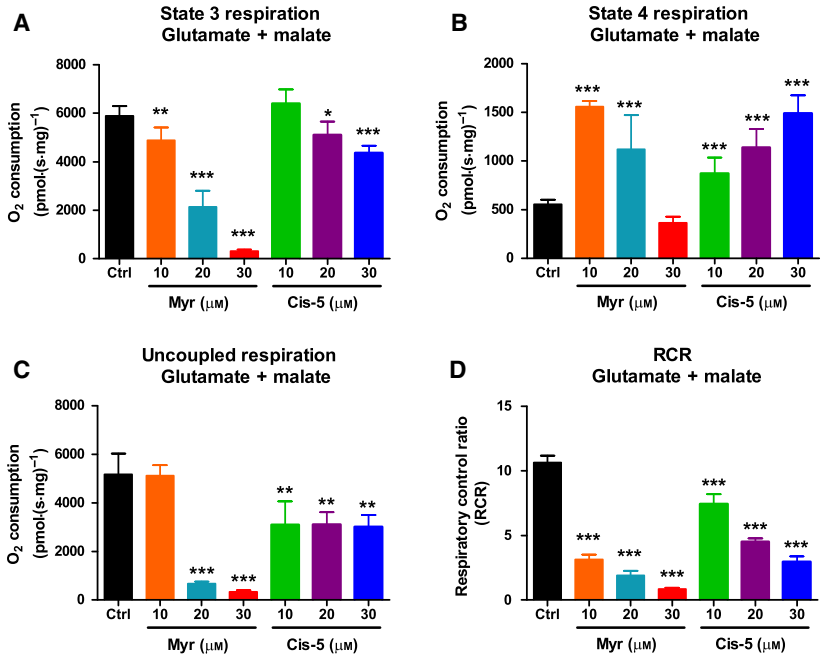

Fig. 4. Effects of Myr and Cis-5 acids on respiratory parameters measured by oxygen consumption in glutamate/malate-supported heart mitochondria. (A) State 3 (ADP-stimulated), (B) state 4 (resting), (C) uncoupled (CCCP-stimulated) respiration and (D) respiratory control ratio (RCR). Glutamate plus malate (2.5 mM each) were used as substrates. Mitochondrial preparations (0.1 mg protein·mL^{-1}) and Myr or Cis-5 (10–30 μM) were added to the incubation medium at the beginning of the assays. Controls were performed in the absence of fatty acids. Values are the mean ± SD of three to four independent experiments (N) and are expressed as pmol O_2·s^{-1}·(mg protein)$^{-1}$. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to controls (Duncan's multiple range test).

destabilization of mitochondrial membranes by interaction of these fatty acids with membrane phospholipids was not involved in the deleterious effects provoked by these fatty acids on mitochondrial functions.

Myr and Cis-5 decrease ATP production by heart mitochondria

We also tested whether Myr and Cis-5 could alter ATP production in heart mitochondrial preparations. Myr and Cis-5 markedly decreased ATP production ($F_{5,23} = 55.431$, $P < 0.001$) (Fig. 9), reflecting a severe disruption of mitochondrial energy production at a magnitude similar to the ATP synthase inhibitor oligomycin.

Myr and Cis-5 decrease oxygen consumption in heart mitochondria using a substrate-uncoupler inhibitor titration (SUIT) protocol

To further evaluate the effects of Myr and Cis-5 on heart mitochondrial respiration, we assessed the respiratory parameters using a SUIT protocol that allows sequential testing of different substrate and coupling states. A concentration of 10 μM Myr was sufficient to markedly decrease state 3 ($F_{2,16} = 16.263$, $P < 0.001$) (Fig. 10A) that reflects OXPHOS capacity using a combination of NADH-linked substrates [pyruvate plus malate plus glutamate (PMG)]. The presence of succinate as the respiratory substrate also significantly minimized Myr-induced effects ($F_{2,16} = 5.543$, $P < 0.05$) (Fig. 10B), corroborating the previous results demonstrating that this fatty acid is more deleterious to heart respiring mitochondria with NADH-linked substrates. Furthermore, uncoupled respiration indicates that the electron transfer system capacity was also decreased with all substrates (PGM plus succinate) ($F_{2,16} = 3.000$, $P < 0.05$) (Fig. 10C). Finally, 10 μM Cis-5 provoked no significant effects.

Myr decreases ADP- and CCCP-stimulated respiration in cardiac muscle fibers and cardiomyocytes

To analyze the effects provoked by Myr and Cis-5 on mitochondrial functions *in situ*, we first tested the influence of these fatty acids (10 μM) on mitochondrial respiration using permeabilized cardiac muscle fibers (saponin). We found that Myr and Cis-5 strongly decreased oxygen consumption in a cocktail substrate-supported medium (glutamate, malate, pyruvate and succinate) stimulated by ADP (state 3) ($F_{2,23} = 22.465$, $P < 0.001$) (Fig. 11A) or by CCCP (uncoupled state) ($F_{2,23} = 16.029$, $P < 0.001$) (Fig. 11B).

We also evaluated various respiratory parameters (state 3, state 4 and uncoupled respiration) measured by oxygen consumption in permeabilized cardiomyocytes (digitonin). Myr and Cis-5 (50 μM) strongly inhibited state 3 and uncoupled respiration in heart cells supported by glutamate plus malate. Furthermore, resting respiration was increased by both fatty

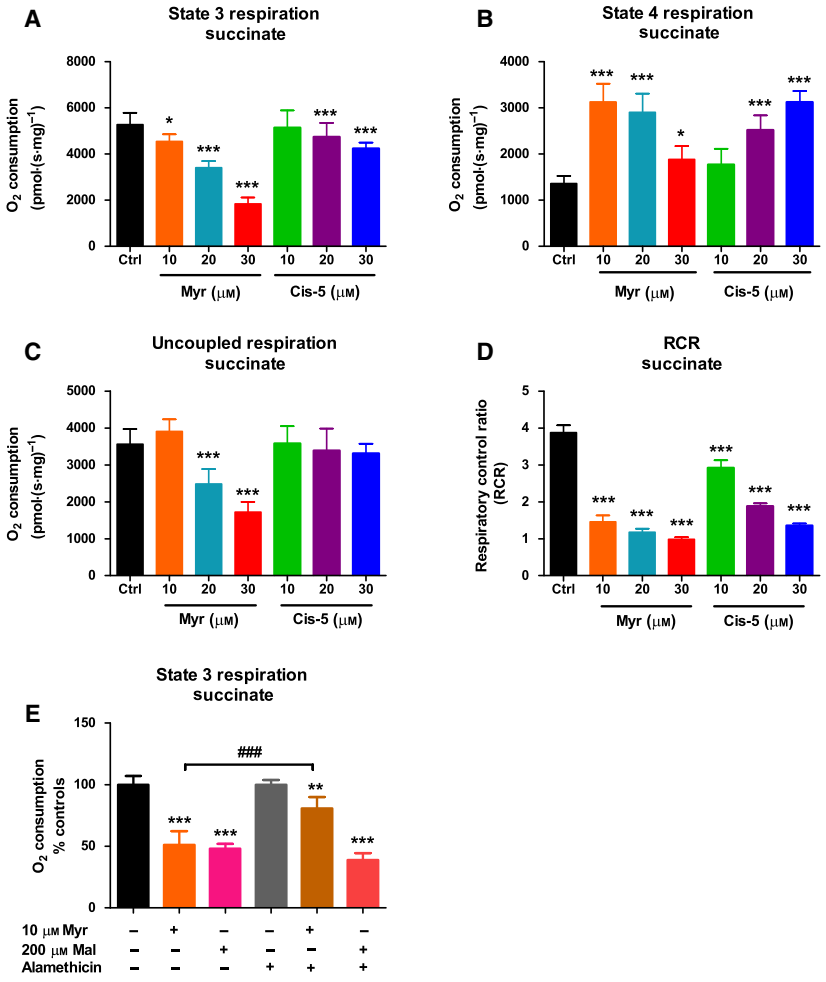

Fig. 5. Effects of Myr and Cis-5 acids on respiratory parameters measured by oxygen consumption in succinate-supported heart mitochondria. (A, E) State 3 (ADP-stimulated), (B) state 4 (resting), (C) uncoupled (CCCP-stimulated) respiration and (D) RCR. Succinate (5.0 mM) was used as substrate. Mitochondrial preparations (0.1 mg protein·mL^{-1}) supplemented by Myr or Cis-5 (10–30 μM) were added to the incubation medium at the beginning of the assays. In some experiments, we used Mal (200 μM) or alamethicin (40 μg·(mg protein)$^{-1}$) in the assay. Controls were performed in the absence of fatty acids. Values are the mean ± SD of three to four independent experiments (N) and are expressed as pmol O_2·s^{-1}·(mg protein)$^{-1}$. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to controls. $^{\#\#\#}P < 0.001$ compared to 10 μM Myr (Duncan's multiple range test).

acids in cultured cardiomyocytes (Fig. 12). The data strongly indicate that these fatty acids behave as metabolic inhibitors in integrated heart cell systems, corroborating our data obtained using mitochondrial preparations.

Myr and Cis-5 do not alter redox homeostasis in the heart

Finally, Myr and Cis-5 at concentrations as high as 100 μM were unable to provoke lipid peroxidation, as determined by malondialdehyde (MDA) levels, or decrease the antioxidant defenses, as measured by reduced glutathione (GSH) levels (Table 2).

Discussion

Energy production from LCFA is severely impaired in VLCAD deficiency. In this regard, clinical features usually develop during episodes of illness or fasting and affect those tissues using LCFA as the primary energy source, such as the heart, liver and skeletal muscle [31]. Patients with VLCAD deficiency usually present severe cardiac manifestations, in which energy deprivation was postulated to play an important role [32]. However, the precise underlying mechanisms of the potentially lethal cardiomyopathy are still poorly established. During crises of metabolic decompensation, there is a large increase in the concentrations of the accumulating metabolites, particularly LCFA and their carnitine derivatives. Furthermore, high levels of these endogenous compounds during these episodes [7,8,33] are associated with a worsening of the symptoms and, more specifically, with cardiac arrhythmias and cardiomyopathy, which can be prevented or attenuated in many patients by early appropriate therapeutic measures, including restriction of LCFA [2,34–36], suggesting that the long-chain acyl-CoA esters and/or LCFA may be cardiotoxic and contribute to the cardiomyopathy in this disorder [37]. This hypothesis is supported by a previous study reporting that the monounsaturated fatty acids, such as *cis*-9-tetradecenoic

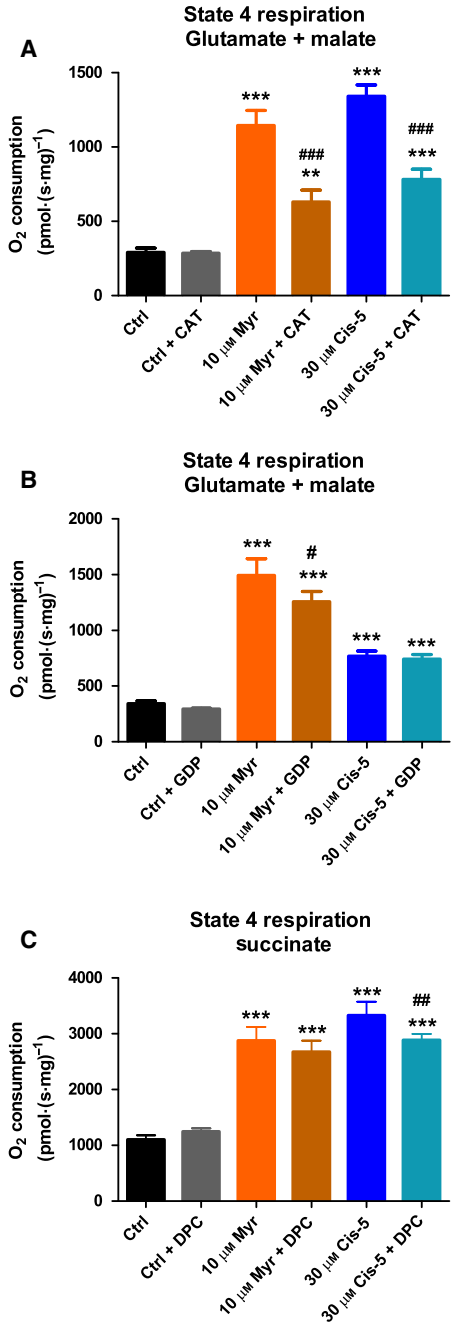

Fig. 6. Effects of Myr and Cis-5 acids on resting (state 4) respiration in the presence of (A) CAT (30 μM), (B) GDP (200 μM) or (C) DPC (100 μM) in heart mitochondria. Glutamate plus malate (2.5 mM each) or succinate (5.0 mM) were used as substrates. Mitochondrial preparations (0.1 mg protein·mL^{-1}) and Myr (10 μM) or Cis-5 (30 μM) were added to the incubation medium at the beginning of the assays. Controls were performed in the absence of fatty acids. Values are the mean ± SD of three to four independent experiments (N) and are expressed as pmol O_2·s^{-1}·(mg protein)$^{-1}$. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to Myr or Cis-5 (Duncan's multiple range test).

and *cis*-9-hexadecenoic acids, but not shorter- or longer carbon-chain fatty acids, disrupt $\Delta\Psi_m$, leading to apoptosis and necrosis of cultured cardiomyocytes [38]. Otherwise, although expanded newborn screening and early treatment decreases mortality and morbidity in most children with VLCAD deficiency, episodes with severe cardiac manifestations cannot be fully prevented in many cases, raising questions about the effectiveness of the present therapy, as well the need for further investigations into the pathogenesis of this disorder [39]. In this context, some observations suggest that impaired mitochondrial energy homeostasis may contribute decisively to the pathophysiology of this disease. These include mitochondrial morphological alterations, rhabdomyolysis and lactic acidemia, which were reported in some affected patients [4,23,24,40], as well as the disruption of mitochondrial bioenergetics in tissues from the genetic animal model with VLCAD deficiency [25,41]. However, the effects of the major accumulating fatty acids, especially Cis-5, which is the biochemical hallmark for the diagnosis of VLCAD deficiency [5], on critical mitochondrial functions had not yet been investigated and were the focus of our attention in the present study.

Therefore, we evaluated the role of Cis-5 and Myr at physiological and pathological concentrations on a large spectrum of mitochondrial bioenergetics measurements and on Ca^{2+} homeostasis in the heart of juvenile rats with the aim of testing whether mitotoxicity could be a contributing factor to cardiac damage in this disease.

Overall, we demonstrated that Cis-5 and Myr significantly compromised bioenergetics by behaving as uncouplers, metabolic inhibitors and MPT inductors in mitochondria isolated from the heart and cardiac muscle fibers of rats. Both fatty acids markedly reduced ATP production, reflecting their severe effects on myocardium bioenergetics. Cis-5 and Myr also impaired mitochondrial Ca^{2+} retention capacity, which represents an important function of mitochondria [42–46], especially with respect to controlling Ca^{2+} homeostasis in the heart, constituting an important regulatory mechanism of cardiac physiology needed for the normal functioning of cardiomyocytes [47].

Our experiments also revealed that Cis-5 and Myr markedly dissipated $\Delta\Psi_m$ in a medium devoid of Ca^{2+}, which may possibly be attributed to an uncoupling effect or, alternatively, to a metabolic inhibition. Furthermore, Cis-5- and Myr-induced $\Delta\Psi_m$ dissipation was magnified in Ca^{2+}-loaded heart mitochondria and fully prevented by RR, a known inhibitor of the mitochondrial Ca^{2+} uniporter [48], as well as by CsA plus ADP [49,50]. Because CsA inhibits MPT by

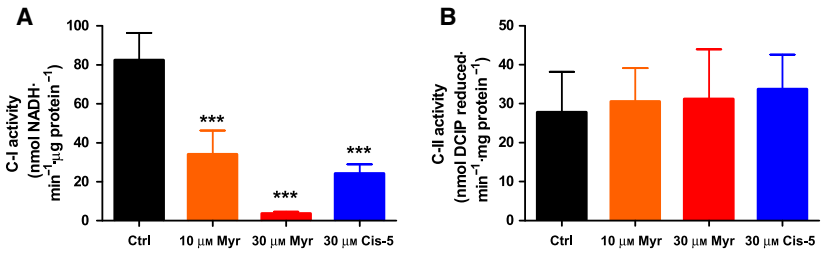

Fig. 7. Effects of Myr and Cis-5 acids on the respiratory chain CI (A) and CII (B) activities in heart mitochondria. Mitochondrial preparations and Myr (10–30 µM) or Cis-5 (30 µM) were pre-incubated for 30 min. Values are the mean ± SD of four to six independent experiments (animals) performed in triplicate and expressed as nmol NADH oxidized·min^{-1}·(mg protein)$^{-1}$ (A) and as nmol DCIP reduced·min^{-1}·(mg protein)$^{-1}$ (B). ***$P < 0.001$ compared to controls (Duncan's multiple range test).

Table 1. Effects of Myr and Cis-5 acids on the activities of GDH, SDH and MDH in heart mitochondria. Mitochondrial preparations and Myr (10–30 µM) or Cis-5 (30 µM) were pre-incubated for 30 min. Values the are mean ± SD of three to six independent experiments (animals) performed in triplicate and expressed as nmol NADH·min^{-1}·(mg protein)$^{-1}$ (GDH), nmol DCIP reduced·min^{-1}·(mg protein)$^{-1}$ (SDH) and µmol NAD·min^{-1}·(mg protein)$^{-1}$ (MDH). No significant differences were detected (one-way ANOVA).

	Control	10 µM Myr	30 µM Myr	30 µM Cis-5
GDH	17.7 ± 2.7	18.9 ± 2.8	19.7 ± 3.9	17.6 ± 2.5
SDH	50.4 ± 10.8	44.2 ± 15.3	54.1 ± 14.8	43.5 ± 5.5
MDH	28.4 ± 5.3	27.8 ± 5.5	25.0 ± 4.7	29.4 ± 7.5

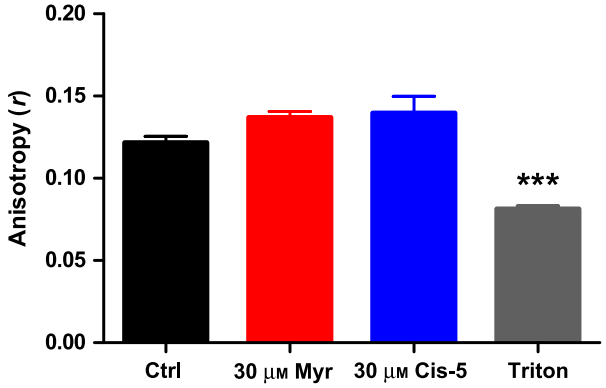

Fig. 8. Effects of Myr and Cis-5 acids on mitochondrial membrane fluidity in heart mitochondria. Glutamate plus malate (2.5 mM each) were used as substrates. Mitochondrial preparations (0.1 mg protein·mL^{-1}) and Myr or Cis-5 (30 µM) were added to the incubation medium at the beginning of the assays. Triton X-100 (0.1%) was also used as a positive control. Controls were performed in the absence of fatty acids. Values are the mean ± SD of four independent experiments (N) and are expressed as fluorescence anisotropy (*r*). ***$P < 0.001$ compared to controls (Duncan's multiple range test).

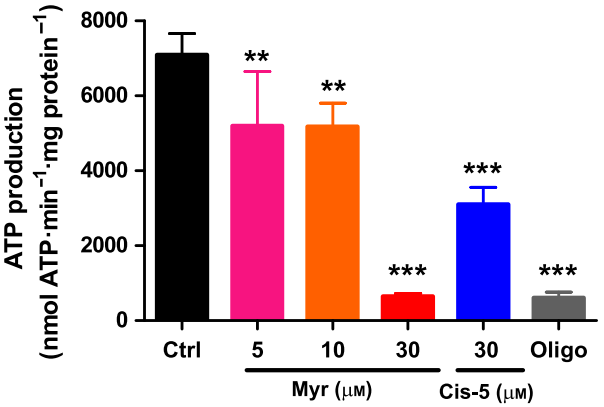

Fig. 9. Effects of Myr and Cis-5 acids on ATP production in heart mitochondria. All experiments were performed in a reaction medium containing heart mitochondrial preparations (0.1 mg protein·mL^{-1}) supported by glutamate plus malate (2.5 mM each). Myr (5–30 µM) or Cis-5 (30 µM) were added at the beginning of the assay. Controls were performed in the absence of fatty acids. Oligomycin A (Oligo) (1 µg·mL^{-1}) was used as a positive control. Values are the mean ± SD of six independent experiments (animals) and are expressed as nmol ATP·min^{-1}·(mg protein)$^{-1}$. **$P < 0.01$ and ***$P < 0.001$ compared to controls (Duncan's multiple range test).

inactivating cyclophilin D, a mitochondrial matrix protein associated with MPT occurrence [51–54], whereas ADP binds to the ANT in the mitochondrial matrix side inhibiting MPT [49,50], these data indicate that Cis-5 and Myr induce MPT pore opening. On the other hand, it is unlikely that oxidative damage to pore proteins as a result of reactive species attack could explain Myr- and Cis-5-induced MPT in Ca^{2+}-loaded mitochondria because, at high concentrations (100 µM), these fatty acids were unable to provoke oxidative stress determined by MDA and GSH levels. This assumption is reinforced by the observations that the potent reducing agent dithiothreitol was unable to prevent Myr-elicited MPT. Furthermore, a change of mitochondrial membrane fluidity that can be associated with MPT induction [55,56] is also unlikely because mitochondrial membrane anisotropy was not changed by Myr and Cis-5. Our present data

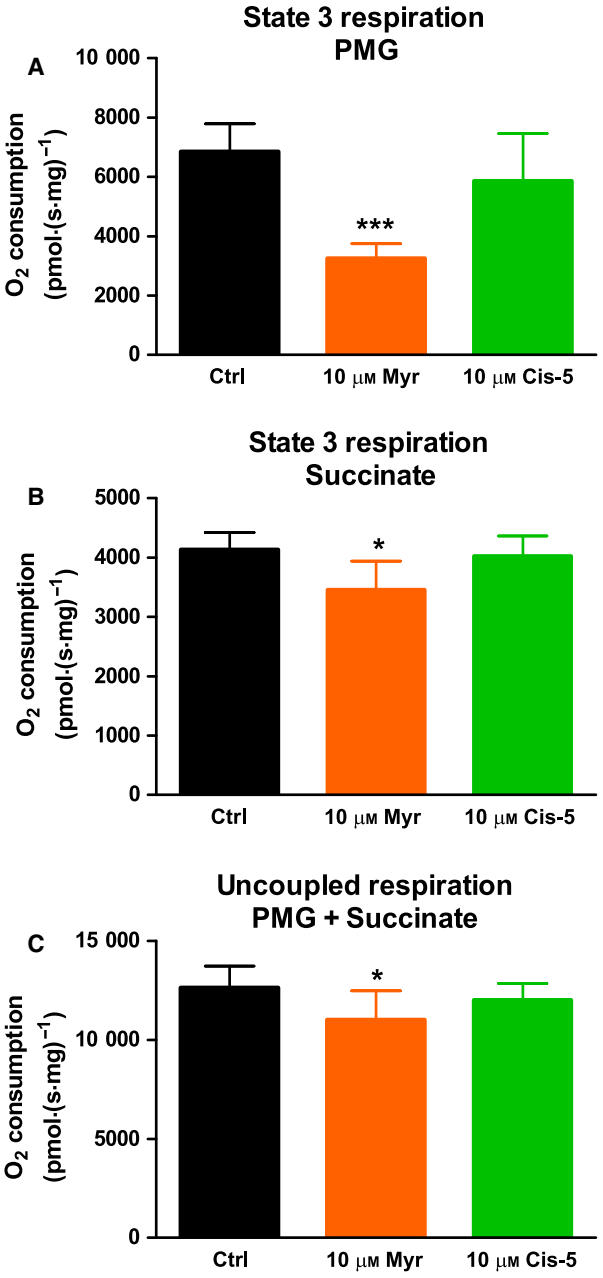

Fig. 10. Effects of Myr and Cis-5 acids on respiratory parameters measured by oxygen consumption in heart mitochondria using the SUIT protocol. (A) CI-linked state 3 (ADP-stimulated), (B) CII-linked state 3 (ADP-stimulated) and (C) CI&II-linked uncoupled (CCCP-stimulated) respiration. Pyruvate (5 mM), malate (0.5 mM) plus glutamate (10 mM) (PMG) and succinate (SUC) (10 mM) were used as substrates. Mitochondrial preparations (0.1 mg protein·mL^{-1}) and Myr or Cis-5 (10 μM) were added to the incubation medium at the beginning of the assays. Controls were performed in the absence of fatty acids. Values are the mean ± SD of five to seven independent experiments (N) and are expressed as pmol O_2·s^{-1}·(mg protein)$^{-1}$. *$P < 0.05$ and ***$P < 0.001$ compared to controls (Duncan's multiple range test).

on the heart are in agreement with previous results showing that Myr also provokes MPT induction in liver mitochondrial preparations [57,58]. However, so far, nothing has been reported about the effects of Cis-5 on these parameters in the heart or in any other tissue.

We cannot rule out that MPT activation could be involved in the decrease in Ca^{2+} retention capacity in Ca^{2+}-loaded heart mitochondria because this condition is shown to induce loss of mitochondrial elements (Ca^{2+}, Mg^{2+}, glutathione, NADH and NADPH), resulting in an impairment of OXPHOS [59–61].

Other findings of the present study revealed that Cis-5 disturbed mitochondrial respiration by increasing resting (state 4) respiration and decreasing RCR, which, allied to the Cis-5-induced nondependent Ca^{2+} $\Delta\Psi_m$ dissipation, is indicative of an uncoupler effect of Cis-5 on OXPHOS. Similar results were obtained with Myr, and this is in accordance with previous studies carried out in the liver and heart [62,63].

ANT was probably involved in the uncoupling behavior of Cis-5 and Myr because CAT attenuated the Cis-5 and Myr-induced increase in state 4 respiration and $\Delta\Psi_m$ dissipation [64–66]. By contrast, UCP proteins and the glutamate/aspartate antiporter were not responsible for the uncoupling behavior caused by Myr and Cis-5 once the respective inhibitors GDP and DPC were unable to significantly alter this effect [67–69]. Thus, it is assumed that the uncoupling effect on OXPHOS could be a result of a protonophoric mechanism with a transbilayer movement of undissociated (linked to protons) Cis-5 and Myr through the mitochondrial inner membrane that is facilitated by ANT [70–72], which is in accordance with previous data on the effects of Myr [73,74]. Finally, we cannot rule out that this uncoupling effect may also have occurred at least in part through the movement of Myr and Cis-5 towards the mitochondrial matrix by other transporters, such as the mono- and tri-carboxylate [75] and the phosphate carriers [76].

Cis-5 and Myr also caused a significant dose-dependent decrease in ADP- and CCCP-stimulated respiration, markedly decreasing ATP synthesis. These data reflect a strong metabolic inhibition caused by these LCFA. Considering that ANT activity is fundamental for ATP production and therefore ADP-stimulated (state 3) respiration, it is suggested that the interaction of these fatty acids with ANT may be involved in their deleterious effects compromising heart bioenergetics. It is also of note that the Cis-5- and Myr-induced decrease in state 3 and uncoupled respiration was higher using NADH (pyruvate, malate and glutamate)

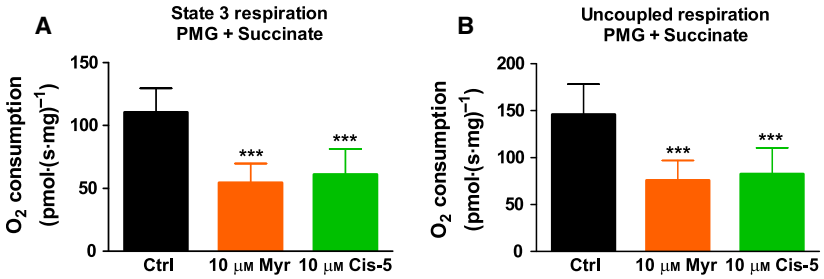

Fig. 11. Effects of Myr and Cis-5 acids on respiratory parameters measured by oxygen consumption in permeabilized myocardial fibers. (A) CI&II-linked state 3 (ADP-stimulated) and (B) CI&II-linked uncoupled (CCCP-stimulated) respiration. Pyruvate (5 mM), malate (5 mM) plus glutamate (10 mM) (PMG) and succinate (SUC) (10 mM) were used as substrates. Approximately 6 mg of permeabilized myocardial fibers and Myr or Cis-5 (10 μM) were added to the incubation medium at the beginning of the assays. Controls were performed in the absence of fatty acids. Values are the mean ± SD of eight to nine independent experiments (N) and are expressed as pmol $O_2 \cdot s^{-1} \cdot mg\ tissue^{-1}$. ***$P < 0.001$ compared to controls (Duncan's multiple range test).

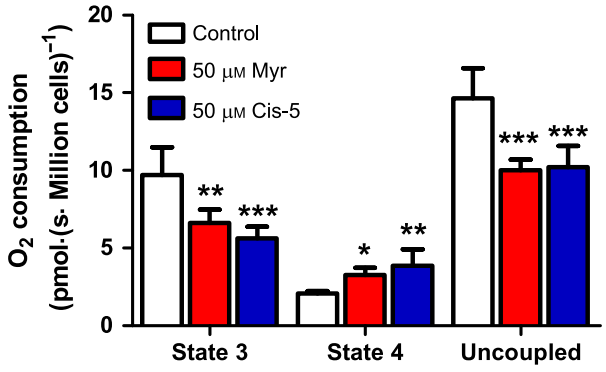

Fig. 12. Effects of Myr and Cis-5 acids on respiratory parameters measured by oxygen consumption in cardiac cells. Glutamate (10 mM) plus malate (5 mM) were used as substrates. Digitonin (5 μM) was used to permeabilize cardiac cells. Approximately 3 million cells and Myr and Cis-5 (50 μM) were added to 2 mL of the incubation medium at the beginning of the assays. Controls were performed in the absence of fatty acids. Values are the mean ± SD of five independent experiments (N) calculated and expressed as pmol $O_2 \cdot s^{-1} \cdot$million cells^{-1}. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to controls (Duncan's multiple range test).

Table 2. Effects of Myr and Cis-5 acids on MDA concentrations and GSH levels in the heart. Values are the mean ± SD of five to seven independent experiments (animals) and are expressed as a percentage of controls. No significant differences were detected (one-way ANOVA).

	Control	100 μM Myr	100 μM Cis-5
MDA	100 ± 23.2	104.0 ± 23.7	97.5 ± 18.5
GSH	100 ± 23.9	86.3 ± 19.9	124.3 ± 31.1

compared to $FADH_2$ (succinate)-linked substrates in heart mitochondria. These results could partly be attributed to the strong and selective decrease in CI activity caused by Cis-5 and Myr, with no alteration of CII through which succinate releases its electrons. Furthermore, GDH, SDH and MDH activities were not inhibited by these fatty acids, implying that oxidation of glutamate and citric acid cycle impairment were not involved in Cis-5 and Myr-induced metabolic inhibition. Other observations showing no Myr-induced decrease in ADP-stimulated respiration in succinate-supported mitochondria permeabilized by alamethicin-mitochondria suggest competition between Myr and succinate for the mitochondrial dicarboxylate carrier, similar to that reported previously in the liver [77].

Mitochondrial matrix NAD(P)H content was also strongly decreased by Cis-5 and Myr in a medium devoid of Ca^{2+}. It is unlikely that the reduction of NAD(P)H occurred via oxidation of the reducing equivalents provoked by reactive species because these fatty acids did not give rise to oxidative stress in the heart. More likely, the reduction of NAD(P)H was a result of the uncoupling behavior of these fatty acids.

Our *in vitro* results achieved in mitochondrial preparations were confirmed by an *in situ* approach performed with intact cell systems consisting of heart fibers and cardiac cells that reliably evaluate mitochondrial functionality within an integrated cellular system [78]. We observed that Cis-5 and Myr severely disturbed heart bioenergetics in cardiac fibers and cardiomyocytes, causing a marked inhibition of oxygen consumption in state 3 and uncoupled respiration.

Notably, the present study highlights the important antioxidant role of the mitochondrial nicotinamide nucleotide transhydrogenase as a result of its ability to regenerate NADPH from NADH (forward mode) [79]. Furthermore, energy deprivation leads to a decrease in the NADH/NAD ratio, reversing the normal direction of this enzyme activity in the heart, consuming NADPH

to form NADH (reverse mode) to support energy metabolism [80] and indirectly favoring MPT pore opening. Accordingly, we cannot exclude the possibility that, under conditions of mitochondrial dysfunction, depletion of NADPH caused by the reverse mode of nicotinamide nucleotide transhydrogenase may potentially induce MPT that is usually triggered by oxidative stress, therefore aggravating the mitochondrial dysfunction caused by Myr and Cis-5. In addition, because uncouplers commonly provoke NADH oxidation, we cannot rule out the possibility that the uncoupling effect of Myr and Cis-5 may also change nicotinamide nucleotide transhydrogenase activity to the reverse mode and its associated harmful consequences.

It is difficult to establish the pathophysiological relevance of our present data because the concentrations of Cis-5 and Myr in the heart of VLCAD-deficient patients are still unknown. However, it should be noted that the mitotoxic effects demonstrated in the present study were achieved with pathological concentrations of accumulating fatty acids similar to those found in the plasma of the affected patients during metabolic decompensation [30], indicating a high vulnerability of heart to the toxicity of these metabolites. Importantly, Myr and Cis-5 were unable to change the evaluated parameters at physiological levels, reinforcing their actions only at pathological concentrations. Furthermore, it is worth noting that the heart mainly utilizes fatty acids for its energy needs, such that the enzymatic fatty acid oxidation steps, including the catabolism of LCFA, are highly expressed in this tissue [21,81]. The present data therefore reinforce the hypothesis that an accumulation of LCFA disturbs crucial mitochondrial functions and may be involved in the clinical phenotype characteristically found in patients affected by VLCAD deficiency. Finally, it is also feasible that the disruption of mitochondrial functions caused by Myr and Cis-5 may be further intensified *in vivo* under stress conditions (e.g. during episodes of metabolic decompensation) in patients affected by VLCAD deficiency.

In conclusion, we provide, for the first time, solid evidence indicating that, at pathological concentrations, Cis-5, which is the principal biochemical marker used for the diagnosis of VLCAD deficiency, markedly disrupts heart mitochondrial energy and Ca^{2+} homeostasis, behaving as a potent metabolic inhibitor, uncoupler and MPT inducer, severely compromising OXPHOS in cardiac muscle from juvenile rats. We also demonstrate that Myr induced a significant decrease in mitochondrial NAD(P)H content and Ca^{2+} retention capacity in mitochondrial preparations, which has not been reported previously. Our *in vitro* data obtained in mitochondrial fractions were confirmed in integrated cell systems (cardiac fibers and cardiomyocytes) that better mimic the cell milieu *in vivo*. Finally, we confirmed the previous deleterious effects of Myr on some parameters of mitochondrial bioenergetics (mitochondrial respiration and $\Delta\Psi_m$). We propose that the mitotoxicity caused by fatty acids accumulating the most in VLCAD deficiency may possibly be associated with the lactic acidemia observed in some affected patients and the disturbance of mitochondrial bioenergetics found in the genetic knockout animal model of VLCAD deficiency [4,23–25,41]. It is tempting to speculate that the findings findings of the present study may be associated with the cardiac manifestations occurring in patients affected by VLCAD deficiency, especially during metabolic crises, which are biochemically characterized by a dramatic increase in accumulating fatty acids. Finally, because Myr and Cis-5 also disturbed skeletal muscle bioenergetics, it could be presumed that mitochondrial dysfunction may contribute towards explaining the lactic acidemia, muscle weakness and severe episodes of rhabdomyolysis observed in patients affected by VLCAD deficiency. Further studies investigating the role of long-chain acylcarnitines and acyl-CoA derivatives found at high concentrations in the blood on mitochondrial homeostasis appear to be justified with respect to shedding more light on the pathophysiology of this disease.

Materials and methods

Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), except Cis-5, which was synthesized by Dr Ernesto Brunet (Universidad Autonoma de Madrid, Madrid, Spain). Stock solutions of Cis-5 and Myr were prepared in ethanol (EtOH). Final concentrations of Myr and Cis-5 in the incubation medium were in the ranges 5–100 μM and 1–100 μM, whereas the EtOH concentration was up to 1%. The same percentage of EtOH was present in controls and was found not to alter *per se* the parameters evaluated.

Animals

Thirty-day-old (juvenile) 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 22 ± 1 °C, with free access to water and 20% (w/w) protein commercial chow (Nuvilab CR-1, Quimtia, Colombo, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, and followed the National Institutes of Health guide for

the care and use of Laboratory animals (NIH Publications No. 8023, revised 1996).

Preparation of mitochondrial fractions

Mitochondria from the heart were prepared according to Ferranti *et al.* [82] with some modifications [28], as well as from skeletal muscle in some experiments, as described previously [83]. The final pellet was resuspended in 10 mM Hepes buffer (pH 7.2) without EGTA containing 225 mM mannitol, 75 mM sucrose and 0.1% BSA (free fatty acid) (heart) or 10 mM Tris buffer (pH 7.4) containing 225 mM mannitol and 75 mM sucrose (skeletal muscle) at an approximate protein concentration of 15 $mg{\cdot}mL^{-1}$. The protein concentration was measured in accordance with the method of Lowry *et al.* [84] using BSA as standard. Mitochondria obtained from the heart and skeletal muscle were used in the assays immediately after isolation and were carried out in the absence or presence of Ca^{2+}.

Preparation of permeabilized myocardial fibers

Cardiac muscle fibers were prepared as described previously [78], with some modifications [83]. Approximately 6 mg of permeabilized ventricle muscle (saponin) was utilized in the assays.

Cell cultures

A heart-derived (ventricular myoblasts, H9C2, number 0098) cell line was obtained from Banco de Células do Rio do Janeiro (Rio de Janeiro, Brazil). Cells were grown and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Culture medium was changed three times a week. Experiments were performed using cells between passages 10 and 14 at the time of the experiments, which were performed in cell cultures with 50–60% confluence [85]. Usually, new cultures were re-established from frozen stocks every 3 months.

Experimental procedures for the spectrofluorimetric assays

Measurements of $\Delta\Psi_m$, NAD(P)H content and Ca^{2+} retention capacity were performed using spectrofluorimetry in a medium containing 150 mM KCl, 5 mM $MgCl_2$, 0.1 $mg{\cdot}mL^{-1}$ BSA, 2 mM KH_2PO_4, 30 μM EGTA, 5 mM Hepes (pH 7.2) and 12 μM Ca^{2+}. Mitochondrial incubations were carried out at 37 °C, with continuous magnetic stirring. The assays were conducted in the presence of ATP synthase inhibitor oligomycin A (1 $\mu g{\cdot}mL^{-1}$) (resting respiration) using heart mitochondria (0.35 mg $protein{\cdot}mL^{-1}$) supported by 2.5 mM glutamate plus 2.5 mM malate. Myr and Cis-5 (10, 20 or 30 μM), $CaCl_2$ (30 μM) and CCCP (3 μM) were added as indicated where appropriate. In some experiments, RR (1 μM), CsA (1 μM) plus ADP (300 μM), CAT (1 μM) or dithiothreitol (5 mM) were added to the incubation medium. Traces are representative of independent experiments carried out in mitochondrial preparations from the hearts of three animals and are expressed as fluorescence arbitrary units (FAU), unless otherwise stated. Statistical analyses were also carried out by quantitatively analyzing the fluorescence changes observed between 150 and 250 s after the beginning of the assays.

$\Delta\Psi_m$

$\Delta\Psi_m$ was estimated as described previously [86,87]. The fluorescence of 5 μM cationic dye safranine O, a $\Delta\Psi_m$ indicator, was followed at excitation and emission wavelengths of 495 and 586 nm. CCCP was added at the end of measurements to abolish $\Delta\Psi_m$. Higher safranine O fluorescence levels reflect lower $\Delta\Psi_m$ values.

Mitochondrial NAD(P)H

Mitochondrial matrix NAD(P)H fluorescence was measured at 340 nm excitation and 450 nm emission wavelengths and CCCP was added at the end of the measurements to induce maximal NAD(P)H oxidation.

Mitochondrial Ca^{2+} retention capacity

Ca^{2+} retention capacity was determined 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 [50]. A low concentration of ADP (10 μM) was present in the incubation medium containing heart or skeletal muscle mitochondrial preparations to achieve more consistent mitochondrial Ca^{2+} uptake responses [50]. Single (30 μM) or successive (5 μM) additions of $CaCl_2$ were used in the experiments. At the end of the measurements, maximal Ca^{2+} release was induced by CCCP.

Respiratory parameters determined via mitochondrial oxygen consumption

The rate of oxygen consumption was measured using an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) in a thermostatically controlled (37 °C) and magnetically stirred incubation chamber [88] with modifications [83]. The assay was performed with mitochondrial preparations $(0.1\ (mg\ protein)^{-1}{\cdot}mL^{-1}$ using 2.5 mM glutamate plus 2.5 mM malate or 5 mM succinate plus 1 μM of the CI inhibitor rotenone as substrates) and incubated in a buffer

containing 0.3 M sucrose, 5 mM KH_2PO_4, 1 mM EGTA, 0.1 mg·mL^{-1} BSA, 5 mM Mops (pH 7.4). Myr and Cis-5 (10, 20 or 30 μM) was added to the reaction medium at the beginning of the assay. State 3 respiration was measured after the addition of 1 mM ADP to the incubation medium and uncoupled respiration after the addition of 1 μM CCCP. To measure resting (state 4) respiration, 1 μg·mL^{-1} oligomycin A was added to the incubation medium. The RCR (state 3/state 4) was then calculated. States 3, 4 and uncoupled are expressed as pmol O_2 consumed·s^{-1}·(mg protein)$^{-1}$. Only mitochondrial preparations with RCR > 3 were used in the experiments. In some experiments, GDP (200 μM), CAT (1 μM), DPC (100 μM), alamethicin (40 μg·(mg protein)$^{-1}$) or Mal (0.2 mM) was added to the mitochondrial preparations.

We also measured oxygen consumption in mitochondrial preparations and permeabilized myocardial fibers in the same medium above at 37 °C, using a SUIT protocol. Pyruvate (5 mM), malate (0.5 mM for mitochondria or 5 mM for fibers) and glutamate (10 mM) (PMG) or succinate (10 mM) were used as substrates to determine CI- or CII-linked respiration, respectively. ADP was added at a final concentration of 400 μM to achieve full OXPHOS capacity. Oligomycin (1 μg·mL^{-1}) was added to reconstitute convergent CI&II-linked leak respiration. Titration with the uncoupler CCCP (0.5–1 μM) was performed to determine electron transfer system capacity [78,89].

The rate of oxygen consumption was also measured in cardiomyocytes. Prior to the assay, the cells were centrifuged for 5 min at 500 ***g*** and resuspended in 20 mM Hepes buffer (pH 7.4) containing 0.11 M sucrose, 10 mM KH_2PO_4, 0.5 mM EGTA, 0.1 mg·mL^{-1} BSA, 60 mM K-lactobionate, 20 mM tarurine and 3 mM $MgCl_2$. The cells were permeabilized with digitonin (5 μM) and incubated with 10 mM glutamate plus 5 mM malate as substrates. Myr and Cis-5 (50 μM) were pre-incubated at 37 °C with the cells for 10 min followed by the addition of ADP (1 mM) to measure state 3 respiration during 5 min. Resting respiration (state 4) was determined after the addition of 1 μg·mL^{-1} oligomycin. Finally, CCCP (1.5 μM) was added as a supplement to the medium and uncoupled respiration was measured.

GDH, SDH and MDH activities

The activities of GDH, SDH and MDH were measured in a SpectraMax M5 microplate spectrofluorimeter (Molecular Devices, San Jose, CA, USA) in the presence of protein heart mitochondria (GDH, 150 μg·mL^{-1}; SDH 50 μg·mL^{-1}; MDH, 2 μg·mL^{-1}) pre-incubated in the presence of Myr or Cis-5 (10 or 30 μM) for 30 min at 37 °C in the technique buffer before the assays. Subsequently, GDH activity was determined as described by Melo *et al.* [90], with SDH activity being determined by the method of Fischer *et al.* [91] and MDH activity as descibed by Kitto [92]. GDH and SDH activities are expressed as nmol·min^{-1}·(mg protein)$^{-1}$ and MDH as μmol·min^{-1}·(mg protein)$^{-1}$.

CI and II activities of the respiratory chain

The activities of respiratory chain CI (NADH:ubiquinone oxidoreductase) and II [succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase] were measured in a SpectraMax M5 microplate spectrofluorimeter in the presence of 200 μg·mL^{-1} (CI) and 50 μg·mL^{-1} (CII) of protein (heart mitochondria) pre-incubated in the presence of Myr or Cis-5 (10 or 30 μM) for 30 min at 30 °C in the technique buffer before the assays. CI activity was assayed as described by Brunmair *et al.* [93] and CII activity was determined according to Fischer *et al.* [91]. The activities of the respiratory chain complexes were calculated as nmol·min^{-1}·(mg protein)$^{-1}$.

ATP production

ATP was determined using the firefly luciferin–luciferase assay system in accordance with the manufacturer's instructions [94,95] with modifications [96]. Heart mitochondrial fractions (0.1 mg protein·mL^{-1}) were incubated in respiring medium containing 0.3 M sucrose, 5 mM Mops, 5 mM KH_2PO_4, 1 mM EGTA and 0.1 mg·mL^{-1} 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. Myr and Cis-5 (5, 10 or 30 μM) was added to the reaction medium at the beginning of the assay. The luminescence was measured in a SpectraMax I3 microplate spectrofluorimeter. In some experiments, oligomycin A was used as a positive control.

Mitochondrial membrane fluidity

Membrane fluidity was evaluated in mitochondrial membranes by fluorescence anisotropy (*r*). Briefly, mitochondrial preparations (0.1 mg of protein) were resuspended in 1 mL of 10 mM Tris–HCl buffer (pH 7.4) and 200 μL of diphenylhexatriene in tetrahydrofuran solution (0.2 mM) was added and incubated at 37 °C for 30 min in the presence of 30 μM. Myr or Cis-5 was added to the reaction medium at the beginning of the assay. Fluorescence was then determined at 365 nm (excitation) and 430 nm (emission) in a SpectraMax M5 microplate spectrofluorimeter equipped with a polarizer system. Fluorescence anisotropy data were calculated as described previously [97,98].

MDA and GSH levels

Determination of MDA and GSH levels was carried out in supernatants from hearts pre-incubated with Myr or Cis-5 (100 μM) for 1 h at 37 °C. The rats were initially

intracardiacally perfused with saline solution during 5 min after being anesthetized with a mixture of ketamine (75 $mg \cdot kg^{-1}$) and xilazine (10 $mg \cdot kg^{-1}$). After perfusion, the heart was rapidly removed and supernatants were obtained as described previously [99].

MDA concentrations were measured in heart supernatants in terms of thiobarbituric acid reactive substances [100] with slight modifications [101]. GSH concentrations were measured as described by Browne and Armstrong [102]. The results are expressed as $nmol \cdot (mg\ protein)^{-1}$.

Statistical analysis

The results are presented as the mean ± SD, with the median being used for statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA), followed by the post-hoc Duncan's multiple range test when multiple comparisons were performed, whereas Student's *t*-test was used for paired samples when a single parameter was compared between two different groups. $P < 0.05$ was considered statistically significant for differences between groups. Only significant *F* values are shown in the text. All analyses were carried out using SPSS, version 19.0 (IBM Corp., Armonk, NY, USA).

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Conflict of interests

The authors declare that they have no conflicts of interest.

Author contributions

CC planned and performed the experiments and analyzed the data. AUA planned the experiments, analyzed the data and wrote the paper. JCS, AW and AZ performed the experiments. SMW prepared the cell cultures. MOVS and LHRS provided technical support and prepared the samples. RFC analyzed data and corrected 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 associated with permeability transition pore opening induced by cis-5-tetradecenoic and myristic acids in liver of adolescent rats

Cristiane Cecatto, Alexandre Umpierrez Amaral, Alessandro Wajner, Simone Magagnin Wajner, Roger Frigério Castilho, Moacir Wajner

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Abstract

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Keywords	VLCAD deficiency, myristic acid, cis-5-tetradecenoic acid, energy and calcium homeostasis, liver mitochondria, mitochondrial permeability transition.
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Corresponding Author	Moacir Wajner
Corresponding Author's Institution	Federal University of Rio Grande do Sul
Order of Authors	Cristiane Cecatto, Alexandre Amaral, Alessandro Wajner, simone wajner, Roger Castilho, Moacir Wajner
Suggested reviewers	Toshiyuki Fukao, Terry Derks, Ute Spiekerkoetter, Alicia Kowaltowski, Jean Bastin

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Disturbance of mitochondrial functions associated with permeability transition pore opening induced by cis-5-tetradecenoic and myristic acids in liver of adolescent rats

Cristiane Cecatto[1], Alexandre Umpierrez Amaral[1,2,3], Alessandro Wajner[1], Simone Magagnin Wajner[4], Roger Frigério Castilho[5], Moacir Wajner[1,2,6*]

[1]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

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

[3]Departamento de Ciências Biológicas, Universidade Regional Integrada do Alto Uruguai e das Missões, Erechim, RS, Brazil.

[4]Departamento de Medicina Interna, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[5]Departamento de Patologia Clínica, Faculdade de Ciencias Médicas, Universidade Estadual de Campinas, Brazil

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

***Corresponding author:** Moacir Wajner, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 – Anexo, CEP 90035-003, Porto Alegre, RS – Brazil. Phone: +55 51 3308-5571, fax: +55 51 3308-5540, e-mail: mwajner@ufrgs.br

Abstract

Patients affected by very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency predominantly present severe cardiac and liver dysfunction that may lead them to death, as well as episodic crises of myopathy with rhabdomyolysis, whose pathogenesis is still poorly known. In this study, we demonstrate for the first time that pathological concentrations (10-60 μM) of cis-5-tetradecenoic acid (Cis-5) and myristic acid (Myr), that most accumulate in VLCAD deficiency, decrease ADP-stimulated and uncoupled respiration, respiratory chain electron flow and ATP production in rat liver mitochondrial preparations. In addition, Cis-5 and Myr increased resting respiration with the involvement of the adenine nucleotide translocator and diminished the respiratory control ratio. These fatty acids also reduced mitochondrial membrane potential (ΔΨm) and Ca^{2+} retention capacity, besides inducing mitochondrial swelling and cytochrome *c* release. Finally, Cis-5 and Myr induced mitochondrial permeability transition (MPT) pore opening in Ca^{2+}-loaded mitochondria, once cyclosporin A totally prevented the reduction of mitochondrial ΔΨm and Ca^{2+} retention capacity, as well as induction of cytochrome *c* release. It was also demonstrated that some of the deleterious effects of Cis-5 and Myr occurred in an integrated cellular system consisting of cultured hepatocytes. The present data strongly indicate that disruption of mitochondrial bioenergetics and Ca^{2+} homeostasis probably associated with MPT and caused by the major fatty acids accumulating in VLCAD deficiency may be involved in the liver dysfunction in the affected patients.

Key words: VLCAD deficiency, myristic acid, cis-5-tetradecenoic acid, energy and calcium homeostasis, liver mitochondria, mitochondrial permeability transition.

Abbreviation list: ANOVA, one-way analysis of variance; ANT, adenine nucleotide translocator; AST, aspartate aminotransferase; BSA, bovine serum albumin; CAC, citric acid cycle; CAT, carboxyatractyloside; CCCP, carbonyl cyanide 3-chlorophenyl hydrazine; CII, complex II; CI-III, complex I-III; Cis-5, cis-5-tetradecenoic acid; CIV, complex IV; CS, citrate synthase; CsA, cyclosporin A; DCIP, 2,6,-dichloroindophenol; DPC, diethyl pyrocarbonate; DPH, diphenylhexatriene; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EGTA, ethylene glycol-bis(2-aminoethyl-ether)-N,N,N’,N’-tetraacetic acid; EtOH, ethanol; FAU, fluorescence arbitrary units; GDH, glutamate dehydrogenase; HEPES, N-[2-hydroxyethyl]piperazine-N′- [2-ethanesulfonic acid]; IDH, isocitrato dehydrogenase; LCFA, long-chain fatty acids; MCT, medium chain triglycerides; MDH, malate dehydrogenase; MOPS, 4-morpholinepropanesulfonic acid; MPT, mitochondrial permeability transition; Myr, myristic acid; OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio; ROS, reactive oxygen species; RR, ruthenium red; SDH, succinate dehydrogenase; UCP, uncoupling protein; VLCAD, very long-chain acyl- CoA dehydrogenase; α-KGDH, α-ketoglutarate dehydrogenase; ΔΨm, mitochondrial membrane potential.

1 Introduction

Very long-chain acyl-CoA dehydrogenase (VLCAD) catalyzes the initial step of mitochondrial β-oxidation of long-chain fatty acids (LCFA) with a chain length of 14 to 20 carbons, which is critical for energy production in mammals, particularly during fasting or periods of increased energy need. After being transported into the mitochondria, exogenous and endogenous LCFA undergo progressive shortening by a series of enzymes, releasing acetyl-CoA, ketone bodies and reducing equivalents for the mitochondrial respiratory chain, therefore providing an energy source for mitochondrial enriched tissues, including heart, liver and skeletal muscle (1, 2).

VLCAD deficiency (OMIM#201475) is a severe life-threatening autosomal recessive disorder of fatty acid β-oxidation caused by a defect in the *ACADVL* gene that encodes the VLCAD enzyme (3). The incidence of VLCAD deficiency is around 1:100,000 live births in high risk population (4, 5), but approaches 1:30,000 when using Tandem mass spectrometry for expanded newborn screening (6). More than 800 cases have been already reported (7).

The diagnosis of this disease is achieved by detecting elevated levels of the carnitine esters tetradecenoyl-L-carnitine (C14:1), tetradecadienyl-L-carnitine (C14:2), tetradecanoyl-L-carnitine (C14) and dodecanoyl-L-carnitine (C12) as well as their corresponding fatty acids, namely cis-5-tetradecenoic (Cis-5), tetradecanoic (myristic-Myr) and dodecanoic acids (7), and may be confirmed by molecular analysis of *ACADVL*. More than a hundred pathogenic mutations have been so far discovered (8).

VLCAD deficiency presents heterogeneous clinical phenotypes, with different severities and ages of onset, predominantly involving cardiomyopathy,

hepatopathy with hepatic steatosis and muscular symptoms associated with rhabdomyolysis (9, 10). A severe early onset cardiac and multi-organ failure often lethally manifests in the first months of life with dilated or hypertrophic cardiomyopathy, arrhythmia, pericardial effusion, hypotonia, hepatomegaly with severe liver abnormalities and hypoglycemia (11). A second clinical phenotype of VLCAD deficiency typically presents during early childhood with hypoketotic hypoglycemia and hepatomegaly with hepatic dysfunction, sometimes associated with rhabdomyolysis, but without cardiomyopathy. Finally, a late-onset episodic myopathic phenotype of VLCAD deficiency, being probably the most common phenotype, manifests with episodic rhabdomyolysis usually provoked by exercise, muscle cramps and/or pain, and/or exercise intolerance (6, 12-14). Catabolic situations precipitate acute symptoms in most patients with these phenotypes, and probably result from accumulation and toxicity of the LCFA that cannot be oxidized in tissues, as well as of the long-chain acyl-CoA and acylcarnitine derivatives (6). Furthermore, postmortem examination of the liver, heart and skeletal muscle of a newborn patient with VLCAD deficiency homozygous for a new missense mutation (R456H) and presenting with hypoglycemia, cardiomyopathy, mild hepatomegaly and slight hypoalbuminemia revealed diffuse lipid accumulation, lobular and portal fibrosis as well as severe macrovesicular steatosis (15). It was proposed that the fatal course of the patient may have resulted from diffuse lipid accumulation in the liver and myocardium.

Treatment is based on avoidance of prolonged fasting and aggressive intervention during intercurrent infections that are catabolic stressors, triggering acute metabolic decompensation and worsening symptomatology, as well as

restriction of long-chain fat and supplementation with medium chain triglycerides (MCT) that can diffuse directly into the mitochondria and bypass the enzyme deficiency (16). The use of carnitine supplementation is controversial (17). The major concern stems from studies in a mouse model of VLCAD deficiency. Mice given L-carnitine supplementation accumulated higher levels of long-chain acylcarnitines that potentially provokes myocardial toxicity (18). Finally, triheptanoin has been used in a few individuals with the goal of providing calories as well as anaplerotic carbons. Formal phase 2 clinical trials of triheptanoin showed potential benefits (19, 20). In addition to these common therapeutic measures, regular follow up of cardiac function and growth parameters are necessary (7).

The pathophysiology of tissue and particularly liver damage in VLCAD deficiency is still unclear, but may be due to energy deprivation since liver essentially utilizes fatty acids for most of its energetic needs. Alternatively liver failure may be due to the toxicity of the LCFA or their long-chain acylcarnitines derivatives that accumulate in this disorder (21-23). Furthermore, lipid peroxidation may also play a key role in the pathophysiology of VLCAD deficiency since reactive oxygen species (ROS) can react with cellular fatty acids, initiating the autopropagative processing of lipid peroxides that are potentially toxic to tissues (24). In this particular, it was shown in a previous study that fasting leads to excessive accumulation of liver lipids in the knockout mice model of VLCAD deficiency and was associated with hepatopathy and upregulation of peroxisomal and microsomal oxidation pathways, causing lipid peroxides and ROS generation (25). Furthermore, recent experimental studies demonstrated that the monocarboxylic LCFA accumulating in VLCAD deficiency

severely impairs mitochondrial bioenergetics in heart of young rat, strengthening the hypothesis that lipotoxicity may play an important role in VLCAD deficiency pathogenesis (26).

However, to the best of our knowledge, there is no work evaluating the role of the major LCFA accumulating in VLCAD deficiency, particularly Cis-5, on mitochondrial functions in the liver. Thus, in the present work we investigated the role of Cis-5 and also Myr on important mitochondrial functions in liver purified mitochondrial preparations from young rats and in permeabilized cultured hepatocytes in the hopes to clarify the underlying mechanisms involved in the pathogenesis of the hepatic dysfunction characteristic of VLCAD deficient patients. We determined the ADP-stimulated (state 3) and resting (state 4) respiration, respiratory control ratio (RCR) and uncoupled respiration, the activities of the respiratory chain complexes I to IV and of the citric acid cycle enzymes, ATP production, mitochondrial membrane fluidity, as well as mitochondrial membrane potential ($\Delta\Psi m$), mitochondrial swelling, cytochrome *c* release and Ca^{2+} retention capacity.

2 Material and methods

2.1 Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Myr and Cis-5 were prepared in ethanol (EtOH) and added to the incubation medium at final concentrations of 1 to 60 µM. The maximal concentration of EtOH in the incubation medium was 1%. The same percentage of EtOH was present in the controls and proved not to alter *per se* the evaluated parameters.

2.2 Animals

Thirty-day-old Wistar rats obtained from our breeding colony were used. The animals were maintained on a 12:12 h light/dark cycle in air-conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow. This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (eighth edition, 2011) and approved by the Ethical Committee of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil (n^o 34888). All animal experiments were carried out in the facilities of the Department of Biochemistry at Federal University of Rio Grande do Sul. All efforts were made to use the minimal number of animals necessary to produce reliable scientific data.

2.3 Mitochondrial preparation

Mitochondrial fractions from liver were prepared according to Mirandola and collaborators (27), with modifications (Hickmann et al., 2015). Animals were sacrificed by decapitation, the liver was removed and homogenized with a glass hand-held homogenizer in ice-cold mitochondrial isolation medium containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (BSA, free fatty acid) and 10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (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 resultant pellet was resuspended in 5 mL of isolation medium without EGTA and centrifuged at 12,000 × *g* for 10 min at 4 °C. The final pellet was resuspended in isolation medium without EGTA at an approximate protein concentration of 30 $mg \cdot mL^{-1}$.

Protein concentration was measured by the method of Lowry et al. (28), using BSA as standard. Mitochondria obtained from liver were used in the assays within two hours after isolation and assays were carried out in the absence or presence of Ca^{2+}.

2.4 Hepatocyte cultures

Hepatocarcinoma (HepG2, number CR0294) cell lines were obtained from Banco de Células do Rio de Janeiro (BCRJ, Rio de Janeiro, Brazil). Cells were grown and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Culture medium was changed three times a week. Experiments were performed using cells with less than 15 passages at the time of the experiments. New cultures were re-established from frozen stocks every 2-3 months (29).

2.5 Respiratory parameters determined through mitochondrial oxygen consumption

The rate of oxygen consumption was monitored using an OROBOROS Oxygraph-2k (Innsbruck, Austria) in a thermostatically controlled (37 °C) and magnetically stirred incubation chamber (30) with modifications (31). Myr or Cis-5 (1–60 μM) was added to the reaction medium (0.3 M sucrose, 5 mM KH_2PO_4, 1 mM EGTA, 0.1 mg . mL^{-1} BSA, 5 mM MOPS, pH 7.4) at the beginning of the assay containing the mitochondrial preparations (0.1 mg $protein^{-1}$. mL^{-1} using 2.5 mM glutamate plus 2.5 mM malate or 5 mM succinate plus 1 μM of the complex I inhibitor rotenone as substrates). State 3 and uncoupled respiration

were induded by 1 mM ADP and 0.5 µM CCCP respectively. To measure resting (state 4) respiration, 1 µg·mL^{-1} oligomycin A was added to the incubation medium and the RCR (state 3/state 4) was then calculated. States 3 and 4, as well as uncoupled respiration, were expressed as pmol O_2 consumed·s^{-1}·mg protein^{-1}. Only mitochondrial preparations with RCR greater than 3 were used in the experiments. In some experiments, guanosine diphosphate (GDP, 200 µM), carboxyatractyloside (CAT, 1 µM) or the glutamate/aspartate antiporter inhibitor diethyl pyrocarbonate (DPC, 100 µM) were added to the assays.

2.6 Respiratory chain complexes I-III, II and IV activities

The activities of respiratory chain complexes I-III (32) was measured in a cuvette spectrophotometer (PG Instruments), whereas a Spectramax M5 microplate spectrofluorimeter was used to determine complexes II (33) and IV activities (34). Liver mitochondrial preparations (C-I-III: 9.5 µg protein.mL^{-1}; C-II: 50 µg protein.mL^{-1}; C-IV: 40 µg protein.mL^{-1}) were pre-incubated in the presence of Myr or Cis-5 (30 µM) for 30 min at 30 °C in the assay buffers before the assays. These respiratory chain activities were calculated and expressed as µmol. min^{-1}. mg protein^{-1}.

2.7 Citric acid cycle enzyme activities

The activities of citrate synthase (CS), isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and aspartate transaminase (AST) were determined in a Spectramax M5 microplate spectrofluorimeter using liver

mitochondrial fractions with distinct amounts of protein according to the enzymatic activities (CS: 0.02 mg protein.mL^{-1}; IDH: 0.2 mg protein.mL^{-1}; α-KGDH: 0.25 mg protein.mL^{-1}; SDH: 0.05 mg protein.mL^{-1}; MDH: 0.009 mg protein.mL^{-1}; AST: 0.07 mg protein.mL^{-1}) that were pre-incubated in the presence of Myr or Cis-5 (30 µM) for 30 min at 37 °C before the assays.

The activities of α-KGDH (35) and IDH (36) were determined following NAD^+ reduction and expressed as nmol NADH. min^{-1}. mg protein^{-1}, whereas MDH activity was measured following NADH oxidation and expressed as nmol NAD^+ oxidized. min^{-1}. mg protein^{-1} (37). Citrate synthase (CS) activity was determined according to Srere (38) by determining DTNB reduction and expressed as µmol. min^{-1}. mg protein^{-1}. SDH activity was assessed according to Fischer and collaborators (39) by determining succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase activity and expressed as µmol. min^{-1}. mg protein^{-1}. AST activity was estimated from the reaction in the direction of aspartate and α-ketoglutarate formation and was expressed as nmol $NADP^+$. min^{-1} . mg protein^{-1}. The assay was coupled to NADPH oxidation by GDH from bovine liver (2 U/mL) (40, 41).

2.8 Mitochondrial respiratory parameters in cultured hepatocytes

The respiratory parameters measured by mitochondrial oxygen consumption were also determined in cultured hepatocytes. Before the assay, the cells were centrifuged for 5 minutes at 500 × *g* and resuspended in 20 mM of HEPES buffer, pH 7.4, containing 0.11 M of sucrose, 10 mM of KH_2PO_4, 0.5 mM of EGTA, 0.1 mg/mL of BSA, 60 mM of K-lactobionate, 20 mM of taurine and 3 mM of $MgCl_2$. The cells (3 million/mL) were permeabilized with digitonin

(15 µM) and incubated with 5 mM pyruvate, 10 mM glutamate, 0.5 mM malate and 10 mM succinate as substrates. Cis-5 and Myr (50 µM) were preincubated at 37°C with the cells for 10 minutes, followed by the addition of ADP (1 mM) to measure state 3 respiration. Resting respiration was determined after the addition of 1 $\mu g.mL^{-1}$ of oligomycin. Data were calculated and expressed as pmol O_2 consumed$\cdot s^{-1}\cdot$mg $protein^{-1}$.

2.9 ATP production

ATP levels were determined by the firefly luciferin–luciferase assay system (42, 43) with modifications (44). Liver mitochondrial fractions (0.1 mg protein . mL^{-1}) were incubated in respiring medium containing 0.3 M sucrose, 5 mM MOPS, 5 mM KH_2PO_4, 1 mM EGTA and 0.1 mg . mL^{-1} 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 with 1 $\mu g.mL^{-1}$ oligomycin and 130 mM $HClO_4$ two minutes afterwards. Myr and Cis-5 (30 µM) was added to the reaction medium in the beginning of the assay. The luminescence was measured in a SpectraMax I3 microplate spectrofluorimeter. In some experiments, oligomycin A was used as the positive control.

2.10 Spectrofluorimetric and spectrophotometric assays

Measurements of ΔΨm and Ca^{2+} retention capacity were performed using spectrofluorimetry, whereas mitochondrial swelling was measured by spectrophotometry in a medium containing 150 mM KCl, 5 mM $MgCl_2$, 0.1 $mg\cdot mL^{-1}$ BSA, 5 mM HEPES, 2 mM KH_2PO_4, 30 µM EGTA, pH 7.2. The assays were conducted at 37 °C with continuous magnetic stirring in the presence of 1

μg·mL^{-1} oligomycin A (resting respiration) using mitochondrial preparations (0.35 mg protein·mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate or 5 mM succinate plus 1 μM rotenone. Cis-5 or Myr (1-60 μM), $CaCl_2$ (30–40 μM) and CCCP (3 μM) were added as indicated by the arrows in the figures. Some experiments were performed in the presence of ruthenium red (RR, 1 μM), cyclosporin A (CsA, 1 μM), ADP (300 μM), resveratrol (100 μM) or coenzyme Q10 (50 μM). Traces are representative of independent experiments carried out in mitochondrial preparations from liver of three to four animals and were expressed as arbitrary units, unless otherwise stated.

2.11 Mitochondrial membrane potential (ΔΨm)

ΔΨm was estimated according to Akerman and Wikstrom (45) and Figueira and collaborators (46). The fluorescence of 5 μM cationic dye safranine O, a ΔΨm indicator, was followed at excitation and emission wavelengths of 495 and 586 nm, respectively. CCCP was added in the end of assays to abolish ΔΨm. The fluorescence changes observed between 150 and 250 seconds after the beginning of the assays were used in the statistical calculations.

2.12 Mitochondrial swelling

Mitochondrial swelling was determined as the decrease in the turbidity of the mitochondrial suspension measured at 540 nm wavelength using a spectrophotometer. A decrease in the turbidity indicates an increase in mitochondrial swelling. The absorbance changes observed between 150 and 250 seconds after the beginning of the assays were used in the statistical calculations.

2.13 Cytochrome c release

After swelling experiments the incubation medium from cuvette was collected and centrifuged at 12,000 × *g* for 10 min at 4 °C in order to sediment mitochondria. Thirty-nine parts of the supernatant received one part of denaturant and was diluted 1:2. The cytochrome *c* content was measured using an ELISA kit (Abcam, Cambridge, UK) in accordance with manufacturer's instructions.

2.14 Mitochondrial Ca^{2+} retention capacity

Ca^{2+} retention capacity was determined 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 (47). In the end of the measurements, maximal Ca^{2+} release was induced by CCCP.

2.15 Mitochondrial membrane fluidity

Membrane fluidity was evaluated in mitochondrial membranes by fluorescence anisotropy (r). Mitochondrial preparations (0.1 mg protein) were first resuspended in 1 mL of 10 mM Tris–HCl pH 7.4 buffer. Then, 200 μL of diphenylhexatriene (DPH) prepared in tetrahydrofuran solution (0.2 mM) was added to the reaction medium and incubated at 37 °C for 30 min in the presence of 30 μM Cis-5 or Myr. Fluorescence was then determined at 365 nm (excitation) and 430 nm (emission) in a SpectraMax M5 microplate spectrofluorimeter equipped with a polarizer system. Fluorescence anisotropy data were finally calculated as previously described (48, 49).

2.16 Statistical analysis

Results are presented as mean ± standard deviation, unless otherwise stated. Assays were performed in duplicate or triplicate and the mean or median was used for the statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan's multiple comparison test when F was significant. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out using the 19.0 SPSS Statistics software.

3 Results

This study mostly used mitochondrial preparations from rat liver to investigate potential deleterious effects provoked by Cis-5 and Myr, at concentrations found in plasma of VLCAD deficient patients (10-60 µM) (50), on mitochondrial functions. Some experiments also utilized permeabilized cultured hepatocytes that represent a more physiological cell system.

3.1 Cis-5 and Myr impair mitochondrial respiration in liver

We first determined the influence of Cis-5 and Myr on respiratory parameters assessed by the rate of oxygen consumption using glutamate plus malate or succinate as substrates (Figures 1 and 2). It was first observed that rat liver mitochondria were fully coupled, as indicated by the higher respiratory rates in the presence of ADP (state 3), as compared to those obtained after the addition of the ATP synthase inhibitor oligomycin A (state 4), at control conditions. Furthermore, Cis-5 and Myr at pathological (10-60 µM), but not at physiological concentrations (less than 10 µM), increased up to 3-fold state 4

respiration in a dose-dependent manner regardless of the substrates used [Fig. 1B: $F_{(9,45)}$=58.32, P<0.001. Fig. 2B: $F_{(6,21)}$=36.61, P<0.001] implying an uncoupling effect of these fatty acids on oxidative phosphorylation (OXPHOS). Furthermore, ADP-stimulated (state 3) and CCCP-induced (uncoupled) respiration, as well as RCR were significantly decreased by Cis-5 and Myr, in the presence of glutamate plus malate or succinate as substrates [Fig. 1A: $F_{(9,45)}$=12.67, P<0.001; Fig. 1C: $F_{(9,44)}$=13.98, P<0.001; Fig. 1D: $F_{(9,45)}$=97.34, P<0.001; Fig. 2A: $F_{(6,21)}$=5.41, P<0.01; Fig. 2C: $F_{(6,21)}$=13.69, P<0.001; Fig. 2D: $F_{(6,21)}$=74.90, P<0.001]. It is also clear from the figures that Myr provoked more pronounced effects as compared to Cis-5 at the same doses and that assays employing succinate as the respiring substrate revealed less intense effects. These experiments suggest that Cis-5 and Myr also behave as metabolic inhibitors, decreasing the efficiency of mitochondrial respiration.

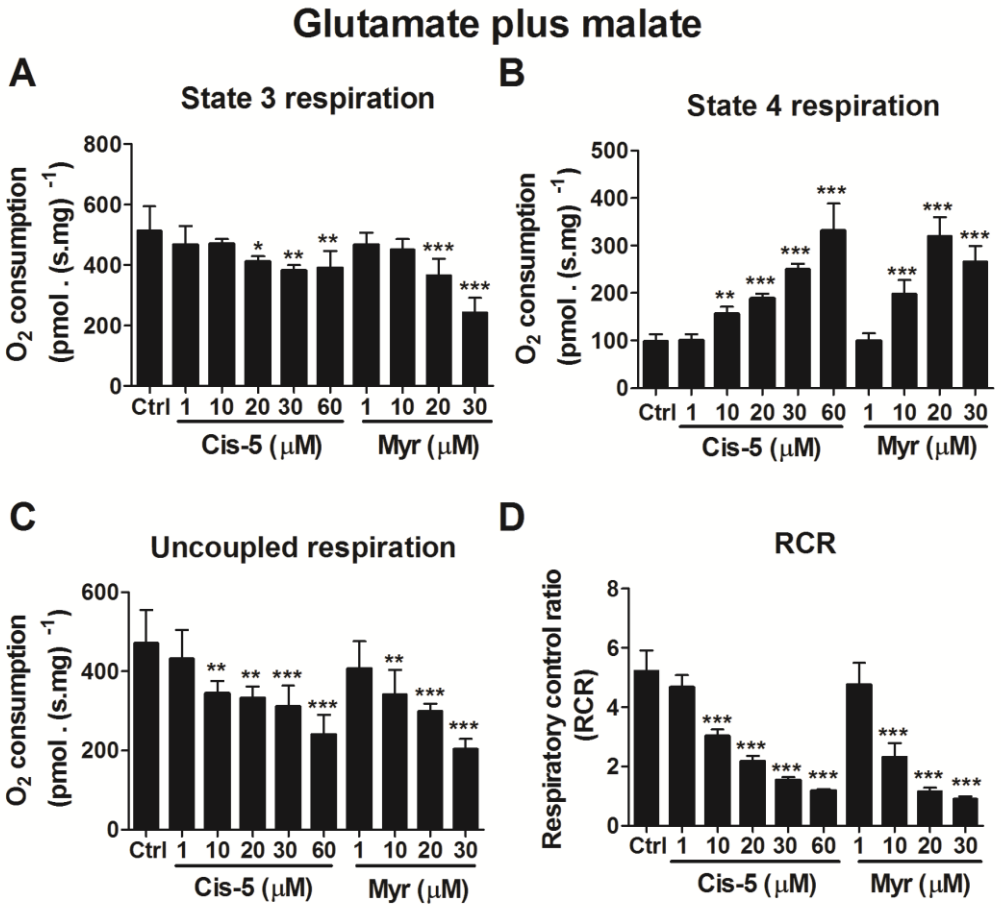

Fig. 1 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on respiratory parameters measured by oxygen consumption in glutamate/malate-supported liver mitochondria. (A) State 3 (ADP-stimulated), (B) state 4 (resting), (C) uncoupled (CCCP-stimulated) respiration and (D) respiratory control ratio (RCR). Glutamate plus malate (2.5 mM each) were used as substrates. Mitochondrial preparations (0.1 mg protein. mL^{-1}) and Cis-5 or Myr (1-60 µM) were added to the incubation medium in the beginning of the assays. Controls were performed in the absence of fatty acids. Values are means ± standard deviation of four to six independent experiments (N) and are expressed as pmol O_2. s^{-1}. mg of $protein^{-1}$. *P < 0.05, **P < 0.01, ***P < 0.001, compared to controls (Duncan multiple range test)

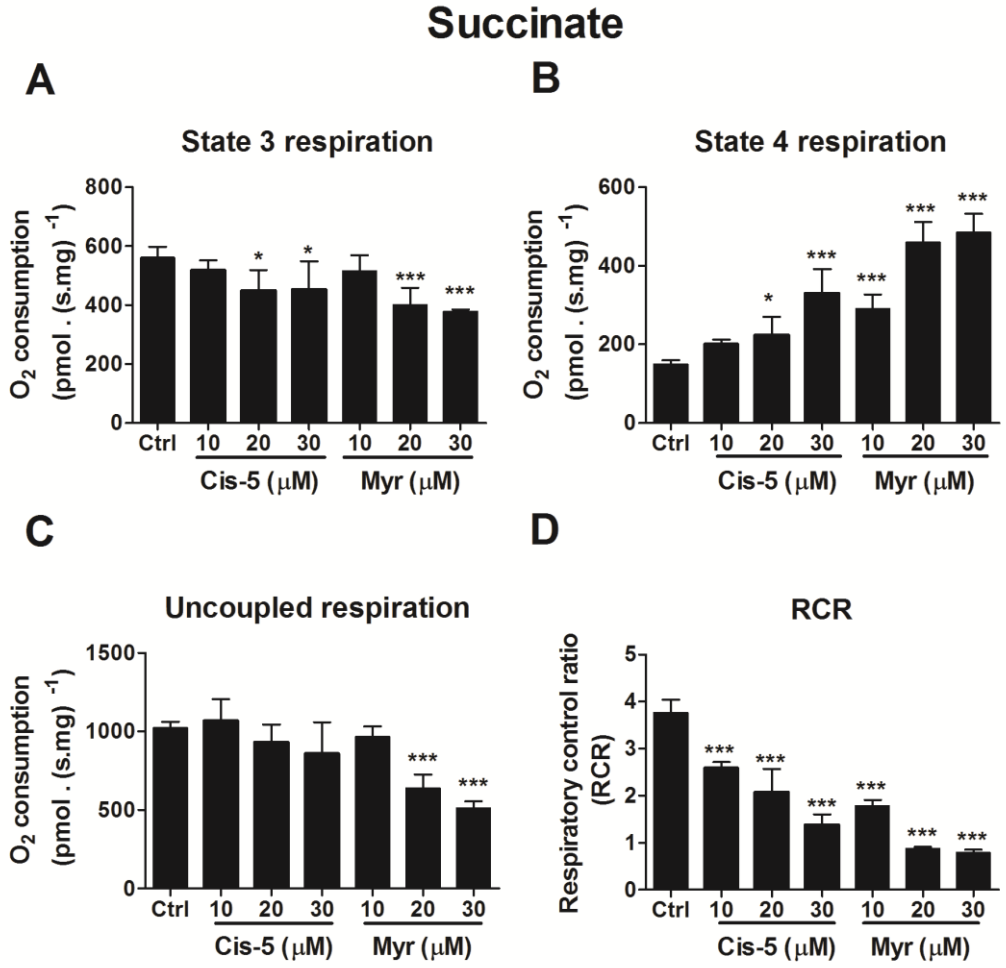

Fig. 2 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on respiratory parameters measured by oxygen consumption in succinate-supported liver mitochondria. (A) State 3 (ADP-stimulated), (B) state 4 (resting), (C) uncoupled (CCCP-stimulated) respiration and (D) respiratory control ratio (RCR). Succinate plus rotenone (5 mM and 1 µM, respectively) were used as substrates. Mitochondrial preparations (0.1 mg protein. mL^{-1}) and Cis-5 or Myr (10-30 µM) were added to the incubation medium in the beginning of the assays. Controls were performed in the absence of fatty acids. Values are means ± standard deviation of four independent experiments (N) and are expressed as pmol O_2. s^{-1}. mg of $protein^{-1}$. *P < 0.05, ***P < 0.001, compared to controls (Duncan multiple range test)

Further results performed under ideal conditions suggested the involvement of the adenine nucleotide translocator (ANT) in Cis-5 and Myr-induced uncoupling effects, since the increase of glutamate plus malate-

supported resting respiration (uncoupling behavior) caused by these fatty acids was attenuated by the ANT inhibitor CAT (Figure 3A) [$F_{(5,18)}$=77.16, P<0.00] but not by the UCP inhibitor GDP (Figure 3B) [$F_{(5,18)}$=10.52, P<0.001] or by the glutamate/aspartate antiporter inhibitor DPC (Figure 3C) [Fig. 3C: $F_{(5,18)}$=30.45, P<0.001].

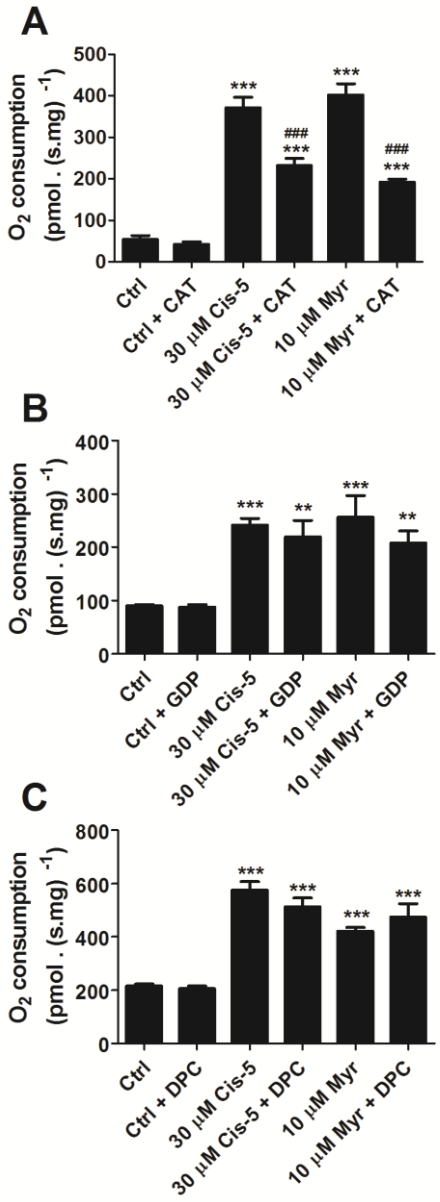

Fig. 3 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on resting (state 4) respiration in the presence of (A) carboxyatractyloside (CAT, 30 μM), (B) guanosine diphosphate (GDP, 200 μM) or (C) diethyl pyrocarbonate (DPC, 100 μM) in liver mitochondria. Glutamate plus malate

(2.5 mM each) (A and B) or succinate plus rotenone (5 mM and 1 µM, respectively) (C) were used as substrates. Mitochondrial preparations (0.1 mg protein. mL^{-1}) and Cis-5 (30 µM) or Myr (10 µM) were added to the incubation medium in the beginning of the assays. Controls were performed in the absence of fatty acids. Values are means ± standard deviation of four independent experiments (N) and are expressed as pmol O_2. s^{-1}. mg of $protein^{-1}$. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls; $^{###}P < 0.001$, compared to Cis-5 or Myr (Duncan multiple range test)

3.2 Cis-5 and Myr reduce complex I-III and α-ketoglutarate dehydrogenase (α-KGDH) activities in mitochondrial preparations

Since the observed effects provoked by these fatty acids on the respiratory parameters in NADH-supported mitochondrial preparations were more pronounced as compared to those of succinate-respiring mitochondria, it is possible that the activities of the respiratory chain complex I and/or of citric acid cycle (CAC) enzymes responsible for the oxidation of glutamate and malate could be inhibited by these compounds. It can be seen in table I that Cis-5 and Myr (30 µM) markedly decreased complex I-III activity (up to 60%), but in contrast did not change complexes II or IV activities [$F_{(2,12)}$=41.24, P<0.001] (Table I). Furthermore, Cis-5 and Myr significantly decreased α-KGDH activity (up to 22%) [$F_{(2,12)}$=5.81, P<0.05], with no effects on other CAC activities, such as SDH, MDH, CS and IDH, as well as AST activity that is required for glutamate oxidation (Table 1).

Table 1. Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on the activities of complexes I-III, II and IV (CI-III, CII and CIV), α-ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), citrate synthase (CS) and aspartate transaminase (AST) in liver mitochondria.

	Ctrl	30 µM Cis-5	30 µM Myr
Respiratory chain complexes activities			
CI-III	294 ± 48.2	121 ± 30.6***	110 ± 25.3***
CII	20.9 ± 3.7	19.9 ± 3.2	17.9 ± 1.1
CIV	8.6 ± 1.1	9.3 ± 3.1	8.0 ± 1.0
Citric acid cycle activities			
α-KGDH	28.1 ± 2.5	21.8 ± 4.3*	22.5 ± 2.4*
SDH	61.9 ± 9.4	71.8 ± 10.8	69.8 ± 15.6
MDH	4904 ± 397	4249 ± 637	5180 ± 650
IDH	9.3 ± 2.1	12.4 ± 1.7	11.8 ± 1.7
CS	89.1 ± 5.8	86.5 ± 2.4	89.3 ± 3.2
Glutamate oxidation			
AST	722 ± 114	665 ± 91.9	738 ± 132

Mitochondrial preparations were pre-incubated for 30 min with Cis-5 (30 µM) or Myr (30 µM). Values are mean ± standard deviation of four to six independent experiments (animals) performed in triplicate and expressed as µmol . min^{-1}. mg $protein^{-1}$ or nmol . min^{-1}. mg $protein^{-1}$. *P < 0.05, ***P < 0.001, compared to controls (Duncan multiple range test).

3.3 Cis-5 decreases liver mitochondrial respiration in permeabilized hepatic cells

In order to investigate the toxicity of Cis-5 and Myr on mitochondrial bioenergetics in a cell system better mimicking the in vivo condition, we next examined the effects provoked by Cis-5 and Myr (50 µM) on mitochondrial respiration using permeabilized cultured hepatocytes respiring with a cocktail of NADH-linked substrates (pyruvate, malate, glutamate) plus succinate. Cis-5 (50 µM) significantly inhibited ADP-stimulated (state 3) and increased resting (state 4) respiration, whereas Myr (50 µM) markedly increased resting respiration in this system (Figure 4). These data corroborate our results obtained using mitochondrial preparations and strongly indicate that Cis-5 behaves as both a

metabolic inhibitor and an uncoupler of OXPHOS in an integrated liver cell system, whereas Myr has a predominate uncoupling effect using this system [Fig. 4A: $F_{(2,19)}$=5.25, P<0.05; Fig. 4B: $F_{(2,20)}$=50.28, P<0.001].

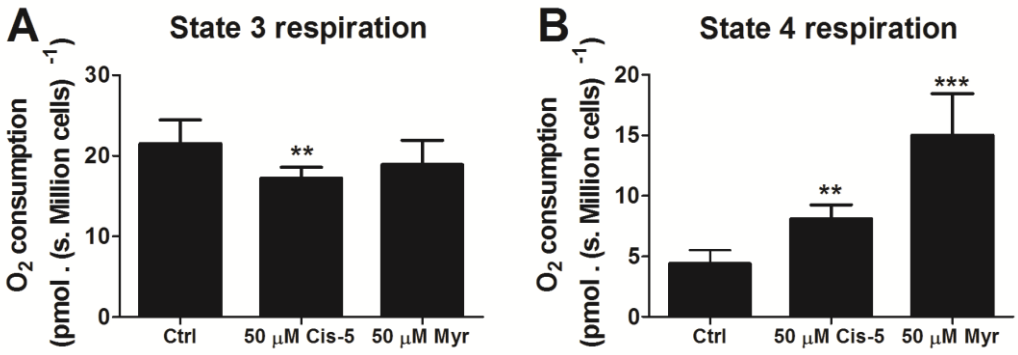

Fig. 4 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on respiratory parameters measured by oxygen consumption in permeabilized hepatocytes. (A) State 3 (ADP-stimulated), (B) state 4 (resting). Pyruvate (5 mM), malate (0.5 mM), glutamate (10 mM) plus succinate (10mM) were used as substrates. Fifteen µM of digitonin were used to permeabilize hepatic cells. Approximately 3 million cells and Myr or Cis-5 (50 µM) were added to 2 mL of the incubation medium in the beginning of the assays. Controls were performed in the absence of fatty acids. Values are means ± standard deviation of seven to nine independent experiments (N) and are expressed as pmol O_2. s^{-1}. mg of protein^{-1}. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test)

3.4 Cis-5 and Myr decrease ΔΨm in liver mitochondria

In order to evaluate whether Cis-5- and Myr-induced disturbance of mitochondrial respiration may alter other mitochondrial functions, we next investigated the influence of Cis-5 and Myr (1-60 µM) on ΔΨm in state 4 (resting) respiring liver mitochondria supported by glutamate plus malate or succinate as substrates in the presence or absence of exogenous Ca^{2+}. It can be seen in figure 5 that Cis-5 and Myr at pathological concentrations significantly decreased ΔΨm in a medium devoid of Ca^{2+} in a dose dependent

manner, corroborating the uncoupling effect observed in the respiratory parameters (state 4 increase). The figure also shows that reduction of ΔΨm was more intense when mitochondrial preparations were challenged by Ca^{2+}. On the other hand, at physiological concentrations these fatty acids were unable to change ΔΨm, stressing their role only at the pathological concentrations characteristic of the affected patients particularly during metabolic decompensation. Furthermore, Cis-5-induced mitochondrial depolarization in the presence of Ca^{2+} was totally prevented by RR that blocks mitochondrial Ca^{2+} uptake and by the classical inhibitors of mitochondrial permeability transition (MPT) CsA and ADP, indicating induction of MPT and a role for Ca^{2+} in this effect. In contrast, the antioxidants resveratrol and coenzyme Q10 were not able to prevent the ΔΨm depolarization caused by Myr, making unlikely the involvement of oxidative attack by reactive oxygen species generation on these effects (fig 5G). ΔΨm dissipation by Cis-5 and Myr also occurred in liver mitochondria supported by succinate as substrate, especially after Ca^{2+} addition (Figure 5) [Fig. 5A: $F_{(5,23)}$=66.12, P<0.001; Fig. 5B: $F_{(5,31)}$=122.38, P<0.001; Fig. 5C: $F_{(3,11)}$=22.86, P<0.001; Fig. 5D: $F_{(3,9)}$=33.79, P<0.001; Fig. 5E: $F_{(4,14)}$=305.46, P<0.001; Fig. 5F: $F_{(4,13)}$=14.15, P<0.001].

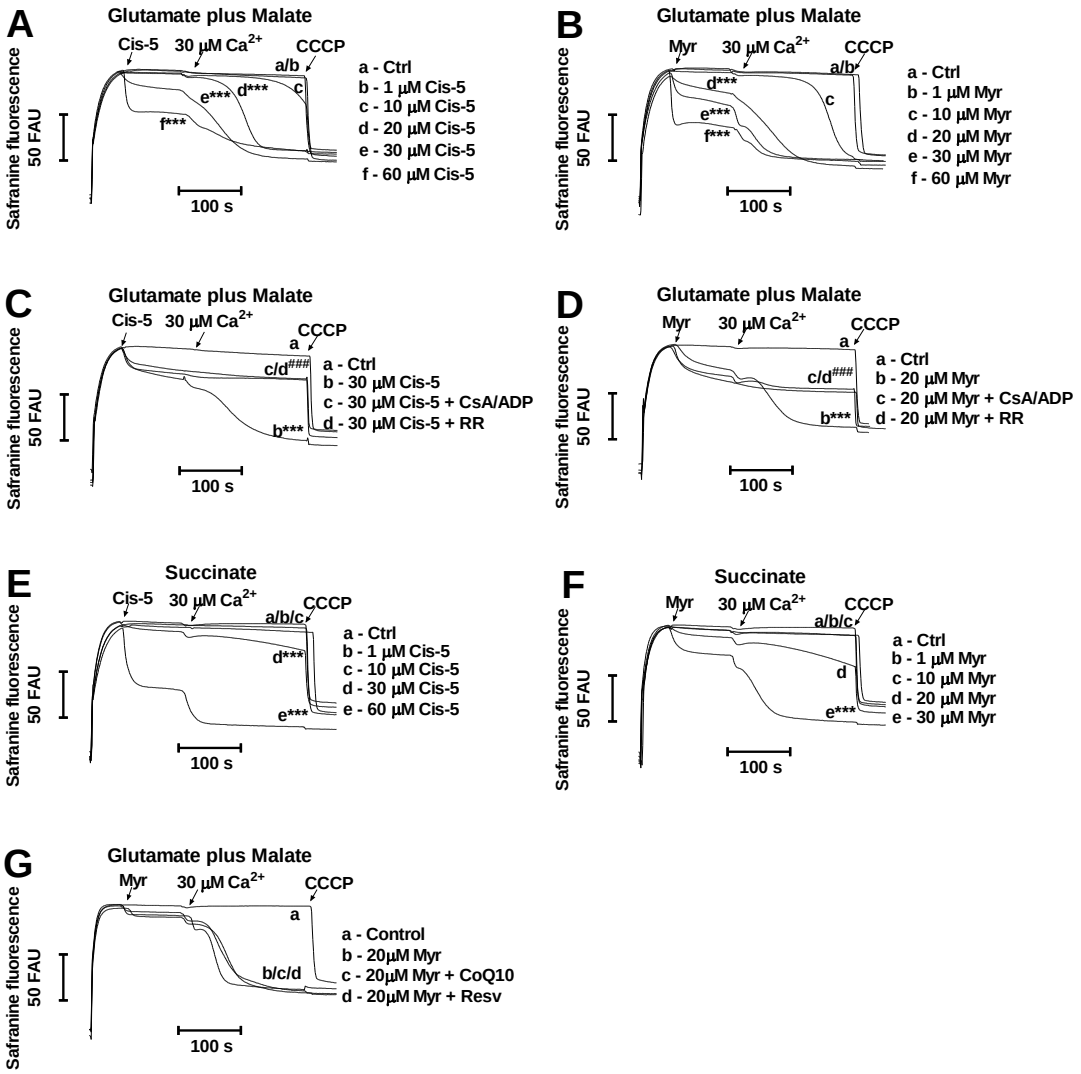

Fig. 5 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on mitochondrial membrane potential in liver mitochondria. All experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein. mL^{-1}) supported by glutamate plus malate (2.5 mM each – panels A-D and G) or succinate plus rotenone (5 mM and 1 μM, respectively – panels E and F). (A and E) Cis-5 (1-60 μM, lines b-f) or (B and F) Myr (1-60 μM, lines b-f) were added 50 seconds after the beginning of the assay. (C) Cis-5 (30 μM, lines b-d). (D and G) Myr (20 μM, lines b-d). Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line c), ruthenium red (RR, 1 μM, line d), coenzyme Q10 (CoQ10, 50 μM, line c) or resveratrol (Resv, 100 μM, line d) were added in the beginning of the assays. All panels refer to mitochondrial preparations before and after addition of 30 μM Ca^{2+}, as indicated. Controls (lines a) were performed in the absence of fatty acids. CCCP was added at the end of the assays. Traces are representative of three independent experiments (N) and

were expressed as fluorescence arbitrary units (FAU). ***P < 0.001 compared to controls, $^{###}P < 0.001$, compared to Cis-5 or Myr (Duncan multiple range test)

3.5 Cis-5 and Myr decrease liver mitochondrial Ca^{2+} retention capacity

Mitochondria have a high capacity to uptake and retain Ca^{2+}, thus buffering intracellular Ca^{2+} concentrations. Therefore, we determined whether Cis-5 and Myr (1-30 µM) could alter Ca^{2+} retention capacity using glutamate plus malate as substrates and a single Ca^{2+} addition. It can be seen in figures 6A and 6B that both fatty acids markedly reduced the mitochondrial Ca^{2+} retention capacity in a dose dependent manner, although Myr effect was stronger as compared to that of Cis-5. We also verified that Cis-5 (30 µM) was not able to change Ca^{2+} retention capacity using succinate as substrate, whereas Myr significantly decreased this parameter (Figure 6C). Finally, CsA totally prevented Myr-induced reduction of mitochondrial Ca^{2+} retention capacity, reinforcing MPT induction by this fatty acid. Similar findings were obtained using multiple additions of Ca^{2+} in glutamate plus malate-supported mitochondria (Figure 6D).

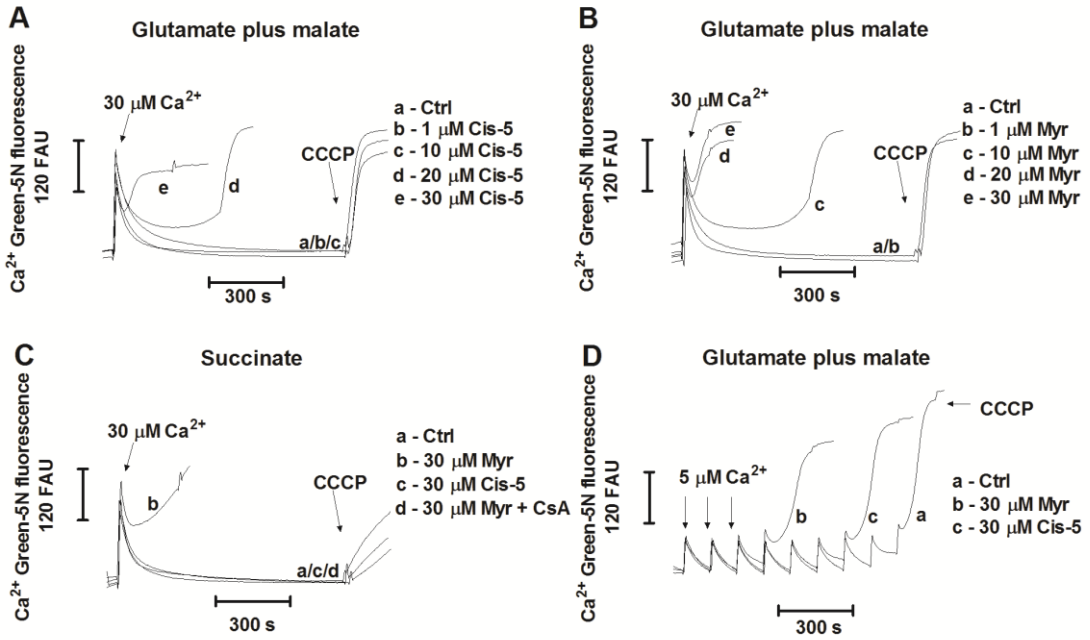

Fig. 6 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on mitochondrial Ca^{2+} retention capacity in Ca^{2+}-loaded liver mitochondria. All experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein. mL^{-1}) supported by glutamate plus malate (2.5 mM each – panels A, B and D) or succinate plus rotenone (5 mM and 1 µM, respectively – panel C). The fatty acids were added in the beginning of the assays. (A) Cis-5 (1-30 µM, lines b-e). (B) Myr (1-30 µM, lines b-e). (C) and (D) Cis-5 or Myr (30 µM, lines b-d). Cyclosporin A (CsA, 1 µM, line d) was added in the beginning of the assay. Panels A-C indicates a single addition of 30 µM Ca^{2+}, whereas in panel D mitochondrial preparations were supplemented by successive additions of 5 µM Ca^{2+} every 2 min, as indicated by the arrows. Controls (lines a) were performed in the absence of these fatty acids. CCCP was added at the end of the assays. Traces are representative of three independent experiments (N) and were expressed as fluorescence arbitrary units (FAU)

3.6 Cis-5 and Myr induce liver mitochondrial swelling and cytochrome c release

Since MPT induction may be associated with mitochondrial swelling and cytochrome *c* release, we tested the effects of Myr and Cis-5 on these parameters. Both LCFA were able to cause swelling in Ca^{2+}-loaded liver mitochondria (Figure 7A and B), although Myr had a more marked effect as compared to Cis-5. It can be also seen that mitochondrial swelling was fully

abolished by CsA plus ADP and RR, further supporting MPT induction (51) [Fig. 7A: $F_{(3,8)}$=48.04, P<0.001; Fig. 7B: $F_{(3,12)}$=570.03, P<0.001]. We also demonstrated that both fatty acids similarly provoked mitochondrial cytochrome *c* release that may have occurred as a consequence of MPT induction and is generally associated with the apoptotic cascade [Fig. 8: $F_{(2,14)}$=3.85, P<0.05] (Figure 8).

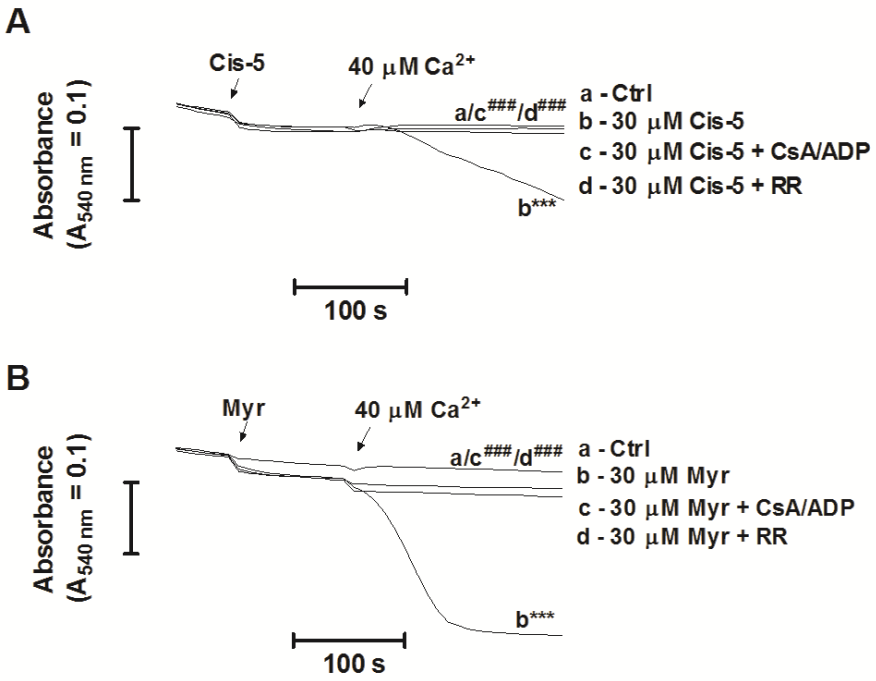

Fig. 7 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on mitochondrial swelling in liver mitochondria. All experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein. mL^{-1}) supported by glutamate plus malate (2.5 mM each). (A) Cis-5 (30 μM, lines b-d) or (B) Myr (30 μM, lines b-d) were added 50 seconds after the beginning of the assays. Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (lines c), ruthenium red (RR, 1 μM, lines d) were added in the beginning of the assays. All panels refer to mitochondrial preparations before and after addition of 30 μM Ca^{2+}, as indicated. Controls (lines a) were performed in the absence of fatty acids. Traces are representative of three independent experiments (N) and were expressed as fluorescence arbitrary units (FAU). ***P < 0.001 compared to controls, $^{###}P$ < 0.001, compared to Cis-5 or Myr (Duncan multiple range test)

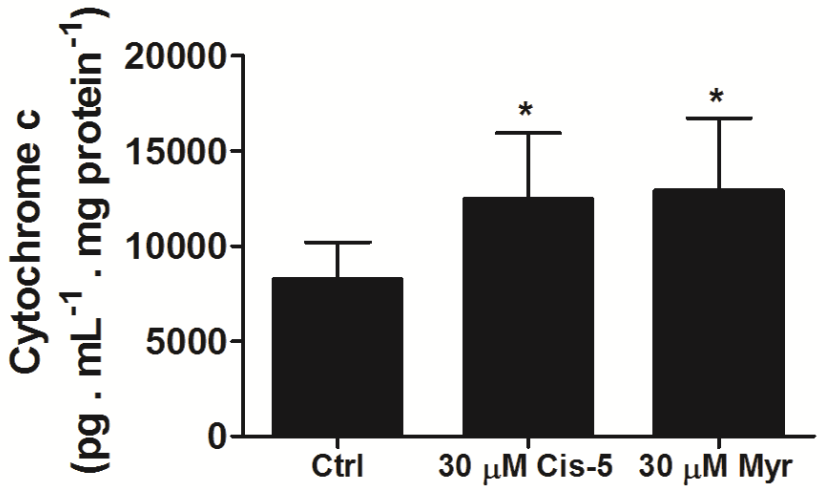

Fig. 8 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on cytochrome *c* release in liver mitochondria. Mitochondria preparations were centrifuged and measurements of cytochrome *c* levels were performed in supernatants fractions after the swelling experiments according to kit manufacturer's instructions. Control was performed in the absence of fatty acids. Values are means ± standard deviation of five to six independent experiments (N) and are expressed as pg cytochrome *c*. mL^{-1}. mg of $protein^{-1}$. *P < 0.05 compared to controls (Duncan multiple range test)

3.7 Cis-5 and Myr do not change mitochondrial membrane fluidity

Since Cis-5 and Myr could potentially interact with mitochondrial membrane lipids altering fluidity and therefore interfering with some of the bioenergetics parameters examined in the present study, such as the uncoupling effect, metabolic inhibition and induction of MPT, we finally evaluated whether Myr and Cis-5 could alter the mitochondrial membrane fluidity by measuring fluorescence anisotropy (r). Triton X-100 (0.1%) was used as a positive control due to its strong detergent activity. No alteration on mitochondrial fluidity was detected, implying that destabilization of mitochondrial membranes by interaction of these fatty acids with membrane phospholipids

was unlikely and probably not involved in the disruption of mitochondrial energy homeostasis [Fig. 9: $F_{(3,20)}$=12.45, P<0.05] (Figure 9).

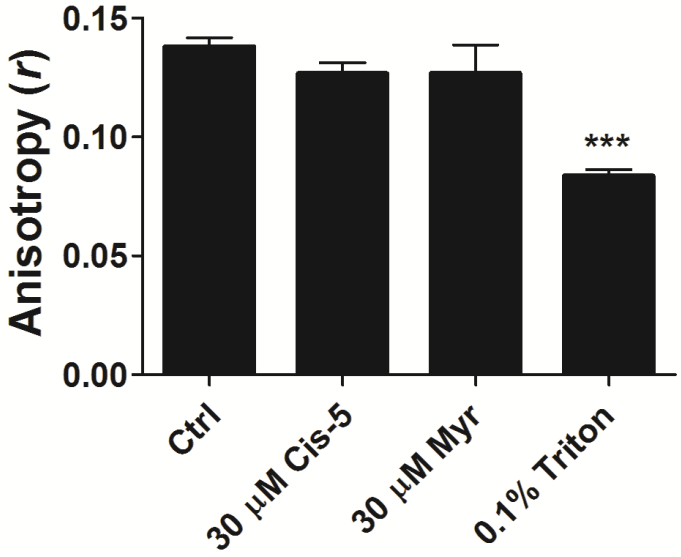

Fig. 9 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on mitochondrial membrane fluidity in liver. Glutamate plus malate (2.5 mM each) were used as substrates. Mitochondrial preparations (0.1 mg protein. mL^{-1}) and Cis-5 or Myr (30 μM) were added to the incubation medium in the beginning of the assays. Triton X-100 (0.1 %) was also used as a positive control. Control was performed in the absence of fatty acids. Values are means ± standard deviation of six independent experiments (N) and are expressed as fluorescence anisotropy (r). ***P < 0.001, compared to controls (Duncan multiple range test)

3.8 Cis-5 and Myr decrease ATP production in liver mitochondria

Considering that respiratory parameters and other mitochondrial bioenergetics parameters were disturbed by Cis-5 and Myr, we further tested whether these LCFA that compromised various mitochondrial functions could impair ATP production in liver mitochondrial preparations. It was verified that Cis-5 and Myr strongly decreased ATP synthesis (up to 70%) at a magnitude similar to the ATP synthase inhibitor oligomycin, reflecting a severe disruption of mitochondrial energy generation. Furthermore, Myr provoked more intense

effects, as compared to Cis-5, which corroborate the other results on mitochondrial bioenergetics [Fig. 10: $F_{(3,10)}$=33.70, P<0.001] (Figure 10).

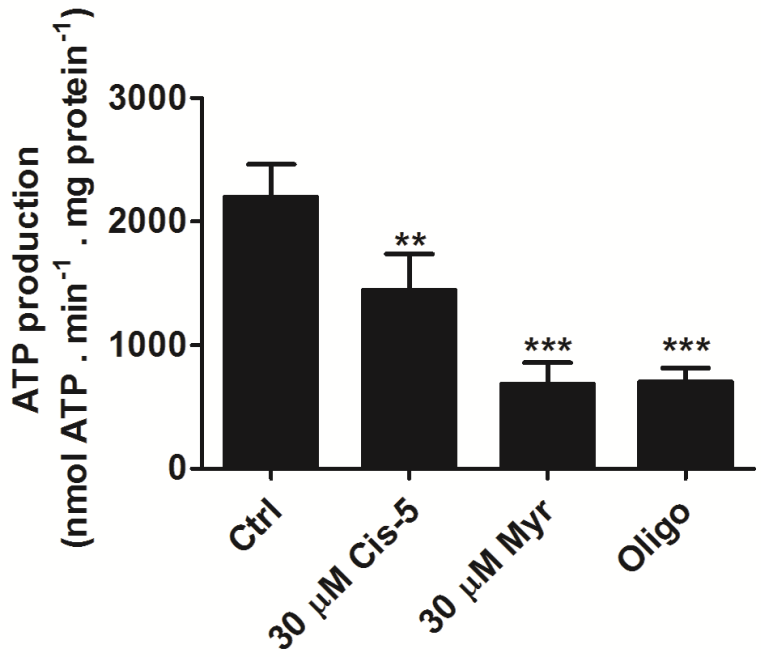

Fig. 10 Effects of cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids on ATP production in liver mitochondria. All experiments were performed in a reaction medium containing liver mitochondrial preparations (0.1 mg protein. mL^{-1}) supported by glutamate plus malate (2.5 mM each). Cis-5 (30 µM) or Myr (30 µM) were added in the beginning of the assay. Controls were performed in the absence of fatty acids. Oligomycin A (Oligo, 1 µg. mL^{-1}) was used as a positive control. Values are means ± standard deviation of four independent experiments (animals) and are expressed as nmol ATP. min^{-1}. mg of $protein^{-1}$. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls (Duncan multiple range test)

4 Discussion

The exact pathomechanisms involved in the severe clinical manifestations of patients with VLCAD deficiency are still poorly established. However, since acute symptomatology, particularly liver failure and cardiac arrhythmias that occur during catabolic crises are associated with marked increases of the concentrations of the accumulating LCFA, it is conceivable that

these compounds may contribute to the pathophysiology of this disorder. Furthermore, humans and mice deficient of VLCAD develop hepatic steatosis and present marked mitochondrial proliferation in heart (52-54), suggesting that mitochondrial dysfunction may underlie at least in part the tissue damage of the affected patients. In this particular, preceding reports demonstrated that long-chain acyl-CoA derivatives inhibit ATP synthesis and oxygen consumption (state 3 respiration) in liver mitochondria (55). Furthermore, Myr was shown to uncouple OXPHOS with the involvement of the dicarboxylate carrier (51) and the ANT (56), as well as to induce CsA-sensitive MPT pore opening in Ca^{2+}-loaded liver mitochondria (57). However, to the best of our knowledge very little has been described regarding the precise underlying mechanisms and signaling pathways of mitochondrial dysfunction in liver and the susceptibility of this tissue to the potential deleterious effects of the fatty acids that most accumulate in VLCAD deficiency, particularly Cis-5. Thus, in the present work we tested the effects of Cis-5 and also Myr on a wide spectrum of important bioenergetics parameters in liver mitochondrial preparations and hepatic cells in the hopes to clarify the mechanisms through which these fatty acids disturb cellular homeostasis.

Overall we found that Cis-5 and Myr, at pathological concentrations (10–60 μM) (50), similar to those observed in plasma of patients with VLCAD deficiency, markedly disturb bioenergetics in liver purified mitochondria and in an integrated cellular system that better mimic the cellular environment (permeabilized hepatocytes). These fatty acids behaved as uncouplers, metabolic inhibitors and MPT inductors, leading to significantly decrease of ATP production, besides impairing mitochondrial Ca^{2+} retention capacity.

We initially observed that Cis-5 and Myr caused a significant reduction of state 3 and uncoupled mitochondrial respiration that was reflected by a severe decrease of ATP generation. The effects were more pronounced especially with glutamate plus malate (NADH-respiring mitochondria as substrates), relatively to succinate ($FADH_2$-respiring mitochondria), suggesting a partial blockage of glutamate oxidation that may have occurred due to inhibition of complex I activity of the respiratory chain, or to reduction of AST activity that is needed for glutamate oxidation, or alternatively due to an enzymatic blockage of the CAC. In this context, we demonstrated a marked inhibition of the respiratory complex I-III activity by Cis-5 and Myr, with no effect on the other complexes of the respiratory chain. Cis-5 and Myr also selectively inhibited α-KGDH activity, without changing the activities of AST, CS, IDH and SDH. Taken together, it is conceivable that these findings may possibly explain at least in part the marked metabolic inhibition of cellular respiration supported by NADH-linked substrates, particularly when glutamate was used as the respiring substrate.

Cis-5 and Myr also increased state 4 respiration and diminished $\Delta\Psi m$ in a dose-dependent manner in a medium devoid of Ca^{2+}, suggesting uncoupling of OXPHOS. Furthermore, the potent ANT inhibitor CAT significantly attenuated Cis-5- and Myr-induced increase of resting respiration (state 4), implying the involvement of ANT in these effects. In contrast, the UCP inhibitor GDP and the glutamate/aspartate antiporter inhibitor DPC did not change the increased resting respiration caused by Cis-5 and Myr, ruling out a role for UCP and the glutamate/aspartate antiporter in these effects. Moreover, since CAT attenuated but not fully prevented the uncoupling effects of Cis-5 and Myr, we cannot rule out the possibility that LCFA anions may also be transferred by

other mitochondrial carriers, such as the mono- and tri-carboxylate carriers (58) as well as the phosphate carrier (59).

It was also observed that ΔΨm dissipation was significantly accentuated when Ca^{2+} was added to the incubation medium and totally prevented by RR, a potent inhibitor of the mitochondrial Ca^{2+} uniporter (60), as well as by CsA plus ADP, classical inhibitors of MPT, supporting a synergistic effect of Ca^{2+}, Cis-5 and Myr, inducing MPT pore opening. It is stressed that CsA inactivates cyclophilin D, a mitochondrial matrix protein associated with the MPT occurrence (61-65), whereas ADP binds to ANT in the mitochondrial matrix inhibiting MPT (47, 66). Otherwise, since MPT induction may result from ΔΨm dissipation (67, 68) (69) or due to metabolic inhibition (70-73), it can be presumed that the uncoupling and the metabolic inhibition effects caused by Cis-5 and Myr may have triggered MPT. In contrast, it is unlikely that disruption of membrane stability was a contributing factor for MPT induction since Cis-5 and Myr did not alter mitochondrial membrane fluidity (74, 75). Oxidative stress mediated by reactive oxygen species generation have also been described as an important condition to induce MPT pore opening (76-78), but this was probably not the case because the antioxidants resveratrol and coenzyme Q10 did not prevent the ΔΨm dissipation caused by Myr.

Other novel findings of the present work were that Cis-5 and Myr induced extensive mitchondrial swelling and cytochrome *c* release in Ca^{2+}-loaded mitochondria presumably a consequence of MPT induction, which is in line with the concept that ΔΨm collapse caused by MPT activation is commonly associated with swelling and loss of mitochondrial elements (Ca^{2+},

Mg^{2+}, glutathione, NADH and NADPH), including proapoptotic factors, such as cytochrome *c*, potentially leading to cell apoptosis and/or necrosis (79, 80).

Another deleterious action provoked by the major LCFA accumulated in VLCAD deficiency (Cis-5 and Myr) was a marked impairment of the mitochondrial capacity to uptake and retain Ca^{2+}, which is an important function of this organelle crucial to cellular Ca^{2+} homeostasis (81-85). It was also observed that this effect was totally prevented by CsA, reinforcing the role of MPT induction. Thus, it is feasible that MPT pore opening could allow Ca^{2+} release from the matrix after reaching a threshold, therefore overcoming mitochondrial Ca^{2+} retention capacity (86, 87). We cannot also exclude the possibility that the disturbed mitochondrial capacity to uptake and retain Ca^{2+} induced by Cis-5 and Myr could also be partially due to the uncoupling effect of these fatty acids, subsequently leading to mitochondrial nonselective permeabilization and thereafter to mitochondrial Ca^{2+} release.

Importantly, the disruption of mitochondrial respiration observed with purified mitochondria were confirmed using hepatic cells that better mimic the *in vivo* human pathological condition. It is also emphasized that our results were obtained under experimental conditions with a low albumin concentration similar to those found in liver cytosol (88), implying that the significant effects caused by Cis-5 and Myr should be attributed to their free active unbinding forms.

Regarding to the pathophysiological relevance of the present findings, although the liver concentrations of free LCFA in patients affected by VLCAD deficiency are still unknown, disturbance of mitochondrial functions were achieved in our study with concentrations of Cis-5 and Myr similar to those found in blood of VLCAD deficent patients (50). In contrast, physiological levels

of these LCFA in plasma (50) did not provoke any alterations of the evaluated parameters, emphasizing the toxicity of pathophysiological concentrations of these accumulating fatty acids. It is also possible that liver concentrations of these metabolites are probably higher inside the hepatic cells of the patients where fatty acid metabolism is acelerated, making this toxicity potentially higher.

5 Conclusion

In conclusion, to the best of our mind we provide for the first time experimental evidence that Cis-5 and Myr, which accumulate at high amounts in VLCAD deficiency, provoke mitochondrial dysfunction by acting as metabolic inhibitors, uncouplers of OXPHOS and inductors of MPT pore opening, besides disturbing cellular Ca^{2+} homeostasis, inducing mitochondrial swelling and cytochrome *c* release, potential indicators of apoptosis. We propose that these deleterious pathomechanisms could exacerbate the energy deprivation caused by the blockage of fatty acid oxidation in the liver and may be associated with the hepatic failure presented by VLCAD deficient patients, particularly during crises.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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CAPÍTULO III

Deregulation of mitochondrial bioenergetics and permeability transition induction caused by major long-chain fatty acids accumulating in VLCAD deficiency in skeletal muscle as potential pathomechanisms of myopathy

Cristiane Cecatto, Alexandre Umpierrez Amaral, Ana Cristina Roginski, Roger Frigério Castilho, Moacir Wajner

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Abstract

Cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids predominantly accumulate in tissues and biological fluids of patients affected by very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency. Most patients commonly manifest myopathy with myalgia, muscular pain and rhabdomyolysis, whose underlying mechanisms are poorly known. Thus, the present study investigated the effects of Cis-5 and Myr on mitochondrial bioenergetics and Ca2+ homeostasis in rat skeletal muscle. Cis-5 and Myr decreased ADP-stimulated (state 3) and CCCP-stimulated (uncoupled) respiration, especially when skeletal muscle mitochondria were supported by NADH-linked as compared to FADH2-linked substrates. In contrast, these fatty acids significantly increased resting respiration (state 4). Similar effects were observed in an integrated cellular system consisting of skeletal muscle fibers therefore validating the data obtained with isolated mitochondria. Furthermore, Cis-5 and Myr markedly decreased mitochondrial membrane potential (ΔΨm) and Ca2+ retention capacity. These effects observed in Ca2+-loaded mitochondria were prevented by cyclosporin A plus ADP and ruthenium red, indicating that Cis-5 and Myr induce mitochondrial permeability transition (MPT). Taken together, our present findings indicate that major long-chain fatty acids accumulating in VLCAD deficiency behave as metabolic inhibitors, uncouplers of oxidative phosphorylation and MPT pore opening inducers in skeletal muscle. It is presumed that these pathomechanisms may contribute to the muscular symptoms and rhabdomyolysis observed in patients affected by VLCAD deficiency.

Keywords	VLCAD deficiency; cis-5-tetradecenoic acid; myristic acid; myopathy; mitochondrial bioenergetics; Ca2+ homeostasis.
Taxonomy	Muscle, Mitochondrion, Lipids
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Corresponding Author	Moacir Wajner
Corresponding Author's Institution	Federal University of Rio Grande do Sul
Order of Authors	Cristiane Cecatto, Alexandre Amaral, Ana Cristina Roginski, Roger Castilho, Moacir Wajner
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Deregulation of mitochondrial bioenergetics and permeability transition induction caused by major long-chain fatty acids accumulating in VLCAD deficiency in skeletal muscle as potential pathomechanisms of myopathy

Cristiane Cecatto[1], Alexandre Umpierrez Amaral[1,2,3], Ana Cristina Roginski[1], Roger Frigério Castilho[4], Moacir Wajner[1,2,5*]

[1]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

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

[3]Departamento de Ciências Biológicas, Universidade Regional Integrada do Alto Uruguai e das Missões, Erechim, RS, Brazil.

[4]Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brazil.

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

***Corresponding author:** Moacir Wajner, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 – Anexo, CEP 90035-003, Porto Alegre, RS – Brazil. Phone: +55 51 3308-5571, fax: +55 51 3308-5540, e-mail: mwajner@ufrgs.br

Abstract

Cis-5-tetradecenoic (Cis-5) and myristic (Myr) acids predominantly accumulate in tissues and biological fluids of patients affected by very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency. Most patients commonly manifest myopathy with myalgia, muscular pain and rhabdomyolysis, whose underlying mechanisms are poorly known. Thus, the present study investigated the effects of Cis-5 and Myr on mitochondrial bioenergetics and Ca^{2+} homeostasis in rat skeletal muscle. Cis-5 and Myr decreased ADP-stimulated (state 3) and CCCP-stimulated (uncoupled) respiration, especially when skeletal muscle mitochondria were supported by NADH-linked as compared to $FADH_2$-linked substrates. In contrast, these fatty acids significantly increased resting respiration (state 4). Similar effects were observed in an integrated cellular system consisting of skeletal muscle fibers therefore validating the data obtained with isolated mitochondria. Furthermore, Cis-5 and Myr markedly decreased mitochondrial membrane potential ($\Delta\Psi m$) and Ca^{2+} retention capacity. These effects observed in Ca^{2+}-loaded mitochondria were prevented by cyclosporin A plus ADP and ruthenium red, indicating that Cis-5 and Myr induce mitochondrial permeability transition (MPT). Taken together, our present findings indicate that major long-chain fatty acids accumulating in VLCAD deficiency behave as metabolic inhibitors, uncouplers of oxidative phosphorylation and MPT pore opening inducers in skeletal muscle. It is presumed that these pathomechanisms may contribute to the muscular symptoms and rhabdomyolysis observed in patients affected by VLCAD deficiency.

Keywords: VLCAD deficiency, cis-5-tetradecenoic acid, myristic acid, myopathy, mitochondrial bioenergetics, Ca^{2+} homeostasis.

1. Introduction

Fatty acid β-oxidation plays a major role in energy production in mitochondrial-enriched tissues such as skeletal muscle. It is therefore conceivable that patients affected by mitochondrial fatty acid oxidation disorders (FAODs) present muscular energy deprivation especially during periods of high-energy demand [1-3]. Very long-chain acyl-CoA dehydrogenase (VLCAD) is responsible for the initial step in mitochondrial β-oxidation of fatty acids, catalyzing the dehydrogenation of long-chain acyl-CoA esters of 12 to 18 carbons at the mitochondrial inner membrane [4, 5]. VLCAD deficiency (OMIM#201475) is the most common mitochondrial β-oxidation defect of long-chain fatty acids, with an incidence of ~1:30,000 to 1:100,000 births [6]. It is a life-threatening autosomal recessive disorder caused by a defect in the *ACADVL* gene [7]. During prolonged fasting, infectious illnesses or physical over-exercise, patients with VLCAD deficiency cannot meet the energy needs of their bodies because of the metabolic blockage and shortage of ketone body and glucose that are rapidly consumed. Symptoms may appear in the neonatal period (early-onset), or later during childhood, adolescence or even adulthood. Early onset severe symptoms are common in the infantile form characterized by high morbidity and mortality with recurrent episodes of hypoketotic hypoglycemia, metabolic acidosis and rhabdomyolysis, especially during fasting and intercurrent illness. Cardiomyopathy with cardiac arrhythmia and hepatopathy with liver failure and hypotonia are frequently observed in this group of patients. Other variants are more common and have a milder disease progression with delayed onset dominated by muscular symptoms (myopathy with myalgia, cramps and rhabdomyolysis). The adult form is characterized by isolated skeletal myopathy generally triggered by exercise or

metabolic decompensation during febrile illness and/or fasting [8]. Mutations in patients with the severe infantile phenotype usually result in total deficiency of VLCAD activity, whereas patients with milder childhood, adolescent and adult phenotypes had mutations with residual enzyme activity [9], suggesting a correlation between genotype and disease severity, although this has been recently disputed [10]. The life-threatening episodes associated with catabolic situations have been attributed to energy deficiency and possibly toxic acylcarnitines accumulation [11].

The diagnosis of VLCAD deficiency is performed by detecting elevated levels of the carnitine esters tetradecenoyl-L-carnitine (C14:1), tetradecadienyl-L-carnitine (C14:2), tetradecanoyl-L-carnitine (C14) and dodecanoyl-L-carnitine (C12), as well as their corresponding fatty acids, namely cis-5-tetradecenoic acid (Cis-5), considered the biochemical hallmark of the disorder, tetradecanoic acid (myristic acid, Myr) and dodecanoic acid in blood [12]. Diagnosis may be confirmed by enzymatic studies and molecular analysis of *ACADVL*.

Treatment is based on reduction of long-chain fat intake supplemented by medium-chain triglycerides, as well as avoidance of fasting. Rapid and aggressive management of infections and hypoglycaemia with high carbohydrate administration preventing metabolic decompensation were shown to decrease mortality [13, 14]. It was recently reported that the use of the anaplerotic triheptanoin substrate that is able to supply the citric acid cycle with adequate amount of energy improve symptomatology of the affected patients [15-18].

This disorder was included in the newborn screening (NBS) programs in many countries [19-21] since morbidity and mortality of VLCAD deficient patients can be reduced by early diagnosis, treatment and surveillance. However, some

long-term complications cannot be totally prevented in patients identified both symptomatically and through NBS, indicating that treatment is still ineffective and more research is needed to elucidate the pathomechanisms of tissue damage [22]. In this particular, mitochondrial abnormalities associated with defects of respiratory chain complexes I-III and II-III and reduction of coenzyme Q levels have been found in skeletal muscle of patients affected by this disease [23], indicating mitochondrial dysfunction.

Therefore, since to the best of our knowledge so far no study was reported in the literature on the pathogenesis of the muscular symptoms that are characteristic of the juvenile and adult forms of VLCAD deficiency, we investigated the role of Cis-5 and Myr, that most accumulate in this FAOD, on important mitochondrial functions, including respiratory parameters measured by oxygen consumption, the activities of the respiratory chain complexes I to IV and ATP synthesis, as well as the mitochondrial membrane potential ($\Delta\Psi m$) and Ca^{2+} retention capacity. For this purpose we used skeletal muscle mitochondrial preparations and fibers from adolescent rats.

2. Material and methods

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Cis-5 was synthesized by Dr Ernesto Brunet (Universidad Autonoma de Madrid, Madrid, Spain). Myr and Cis-5 were prepared in ethanol (EtOH) and added to the incubation medium at concentrations of 1–60 μM. The maximal concentration of EtOH in the incubation medium was 1%. The same percentage of EtOH was present in controls and proved not to alter *per se* the evaluated parameters.

2.2. Animals

Thirty-day-old Wistar rats obtained from our breeding colony were used. The animals were maintained on a 12:12 h light/dark cycle in air-conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow. This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (eighth edition, 2011) and approved by the Ethical Committee of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil (n^{o} 34888). All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data.

2.3. Mitochondrial preparation

Mitochondrial fractions from skeletal muscle (quadriceps) were prepared according to Chweih and collaborators [24], with modifications [25].

Mitochondrial preparations from two quadriceps were used to measure the respiratory parameters state 3, state 4, RCR, uncoupled respiration and ATP

production, whereas four quadriceps were needed for the determination of the activities of the respiratory chain complexes and succinate dehydrogenase, as well as for mitochondrial membrane potential and calcium retention capacity. Protein concentration was determined by the method of Lowry et al. [26], using BSA as standard. Mitochondrial fractions were used in the assays immediately after isolation and assays were carried out in the absence or presence of Ca^{2+}.

2.4. Skeletal muscle fiber preparation

Skeletal muscle fibers were prepared using the plantar muscle as previously described [27] with modifications [25].

2.5. Respiratory parameters determined by oxygen consumption

Oxygen consumption was determined in an OROBOROS Oxygraph-2k (Innsbruck, Austria) as previously reported [25, 28]. Myr and Cis-5 were tested at concentrations ranging from 1 to 60 µM in a medium containing skeletal muscle mitochondrial fractions (0.1 mg protein^{-1}. mL^{-1}) using the following respiring substrates (2.5 mM glutamate plus 2.5 mM malate, 5 mM succinate plus 1 µM of the complex I inhibitor rotenone, 5 mM glutamate or 2.5 mM pyruvate plus 2.5 mM malate). State 3 and uncoupled respiration were measured after addition of 1 mM ADP and 0.5 µM CCCP, respectively, to the incubation medium. Oligomycin A (1 µg·mL^{-1}) was added to measure resting (state 4) respiration. The RCR was then calculated by dividing state 3 per state 4. Respiring parameters were expressed as pmol O_2 consumed. s^{-1} . mg protein^{-1}. We always used mitochondrial preparations with RCR greater than 5. In some experiments alamethicin (Alm, 40 µg . mg protein^{-1}), malonate (Mal, 0.2 mM), resveratrol (Resv, 10 and 100 µM),

coenzyme Q10 (CoQ10, 50 and 100 µM) or creatine (1 mM) were added to the mitochondrial preparations.

We also determined oxygen consumption in permeabilized skeletal muscle fibers according to Kuznetsov [27] with some modifications [25] using 10 mM glutamate and 5 mM malate as substrates. Myr and Cis-5 (30 µM) were pre-incubated at 37 °C for 5 min. State 3, state 4 and uncoupled respiration were expressed as pmol O_2 consumed . s^{-1} . mg $protein^{-1}$.

2.6. Respiratory chain complexes I-III, II and IV activities

The activity of respiratory chain complexes I-III (C I-III) [29] was measured in a cuvette spectrophotometer (PG Instruments), whereas a Spectramax M5 microplate spectrofluorimeter was used to determined complexes II (C II) [30] and IV (C IV) [31] activities. Myr or Cis-5 (30 µM) were pre-incubated in the presence of skeletal muscle mitochondria (C I-III, 30 µg protein . mL^{-1}; C II, 30 µg protein . mL^{-1} and C IV, 15 µg protein . mL^{-1}) for 30 min at 30 °C in each technique buffer (C I-III: 20 mM K_2HPO_4, 20 mM KH_2PO_4, pH 8.0; C II: 62.5 mM K_2HPO_4, 62.5 mM KH_2PO_4, pH 7.4; C IV: 10 mM K_2HPO_4, 10 mM KH_2PO_4, pH 7.0) before the assays. These respiratory chain activities were calculated and expressed as µmol. min^{-1}. mg $protein^{-1}$.

2.7. Succinate dehydrogenase (SDH) activity

The activity of SDH was determined in a Spectramax M5 microplate spectrofluorimeter using skeletal muscle mitochondrial fractions (30 µg protein. mL^{-1}) pre-incubated in the presence of Myr or Cis-5 (30 µM) for 30 min at 37 °C in the technique buffer (62.5 mM K_2HPO_4, 62.5 mM KH_2PO_4, pH 7.4) before the

assays. SDH activity was assessed according to Fischer and collaborators [30] by determining succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase activity and expressed as µmol. min^{-1}. mg $protein^{-1}$.

2.8. ATP production

ATP levels were measured as previously described [32, 33] with modifications [34]. Skeletal muscle mitochondrial preparations (0.1 mg protein . mL^{-1}) were incubated using 2.5 mM malate plus 2.5 mM glutamate as substrates (44) in a total volume of 500 µL. Myr and Cis-5 (30 µM) was added to the reaction medium in the beginning of the assay and luminescence was determined in a SpectraMax I3 microplate spectrofluorimeter.

2.9. Mitochondrial membrane potential (ΔΨm)

ΔΨm was estimated by spectrofluorimetry using mitochondrial preparations (0.35 mg protein·mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate or 2.5 pyruvate plus 2.5 malate according to Akerman and Wikstrom [35] and Figueira and collaborators [36]. Myr or Cis-5 (1-30 µM), $CaCl_2$ (30 µM) and CCCP (3 µM) were added as indicated by the arrows in the figures. In some experiments, ruthenium red (RR, 1 µM), cyclosporin A (CsA, 1 µM), ADP (300 µM), carboxyatractiloside (CAT, 1 µM), *N*-ethylmaleimide (NEM, 10 µM) or melatonin (MEL, 1 mM) were added in the beginning of the assays. The fluorescence of 5 µM cationic dye safranine O, a ΔΨm indicator, was followed at excitation and emission wavelengths of 495 and 586 nm, respectively. The fluorescence changes observed between 150 and 250 seconds after the beginning of the

assays were used in the statistical calculations and expressed as fluorescence arbitrary units.

2.10. Mitochondrial Ca^{2+} retention capacity

Ca^{2+} retention capacity was also determined by spectrofluorimetry using mitochondrial preparations (0.35 mg protein·mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate. Extramitochondrial free Ca^{2+} levels using 0.2 μM Calcium Green-5N (Molecular Probes, Invitrogen, Carlsbad, CA) were measured at excitation and emission wavelengths of 506 and 532 nm, respectively [37]. CCCP was supplemented at the end of the assays for maximal Ca^{2+} release.

2.11. Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical analysis. Results were analyzed by one-way analysis of variance (ANOVA) followed by the post-hoc Duncan's multiple comparison test when F was significant. Only significant F values appear in the text. $P < 0.05$ was considered significant. All analyses were carried out using the 19.0 SPSS Statistics software.

3. Results

3.1 Myristic (Myr) and Cis-5-tetradecenoic (Cis-5) acids impair mitochondrial respiration in skeletal muscle mitochondria

We first tested the effects of Myr and Cis-5 on respiratory parameters measured by oxygen consumption in skeletal muscle mitochondrial preparations using glutamate plus malate (NADH-linked) as substrates. Both fatty acids (10-60

μM) significantly decreased ADP- (state 3), CCCP-stimulated (uncoupled) respiration and RCR, and increased resting respiration (state 4) in a dose dependent manner (Fig. 1A to D) [1A: $F_{(8,39)}$ = 75.23, P<0.001; 1B: $F_{(8,39)}$ = 3.38, P<0.001; 1C: $F_{(8,36)}$ = 78.96, P<0.001; 1D: $F_{(8,39)}$ = 335.49, P<0.001]. Furthermore, a stronger inhibition of state 3 and uncoupled respiration was observed when only glutamate was used as the substrate (Fig. 1E and F) [1E: $F_{(4,16)}$ = 63.12, P<0.001; 1F: $F_{(4,18)}$ = 35.36, P<0.001]. However, when succinate plus rotenone were used as substrates ($FADH_2$-linked), the inhibitory effects were less intense (Fig. 2A and B) [2A: $F_{(8,33)}$ = 10.39, P<0.001; 2B: $F_{(8,33)}$ = 4.27, P<0.01]. It was also seen that Myr-induced inhibition of state 3 was partially prevented in alamethicin-permeabilized mitochondria (Fig. 2C) [2C: $F_{(5,40)}$ = 43.28, P<0.001], suggesting a possible competition between Myr and succinate for the same dicarboxylate transporter [38, 39]. In these experiments, Mal (0.2 mM), a known succinate dehydrogenase inhibitor, was used as a control. We also found that SDH activity was not altered by both fatty acids (Fig. 2D), ruling out a role for an inhibition of this enzyme in the decreased ADP-stimulated and uncoupled respirations. We finally tested the effects of Myr and Cis-5 on oxygen consumption using pyruvate plus malate as substrates (NADH-linked). It can be seen in figure 3 that the inhibitory effect of Myr on state 3 was similar to that observed when using glutamate plus malate as substrates and that Cis-5 provoked milder effects as compared to Myr [3A: $F_{(4,20)}$ = 24.64, P<0.001; 3B: $F_{(4,20)}$ = 19.35, P<0.001].

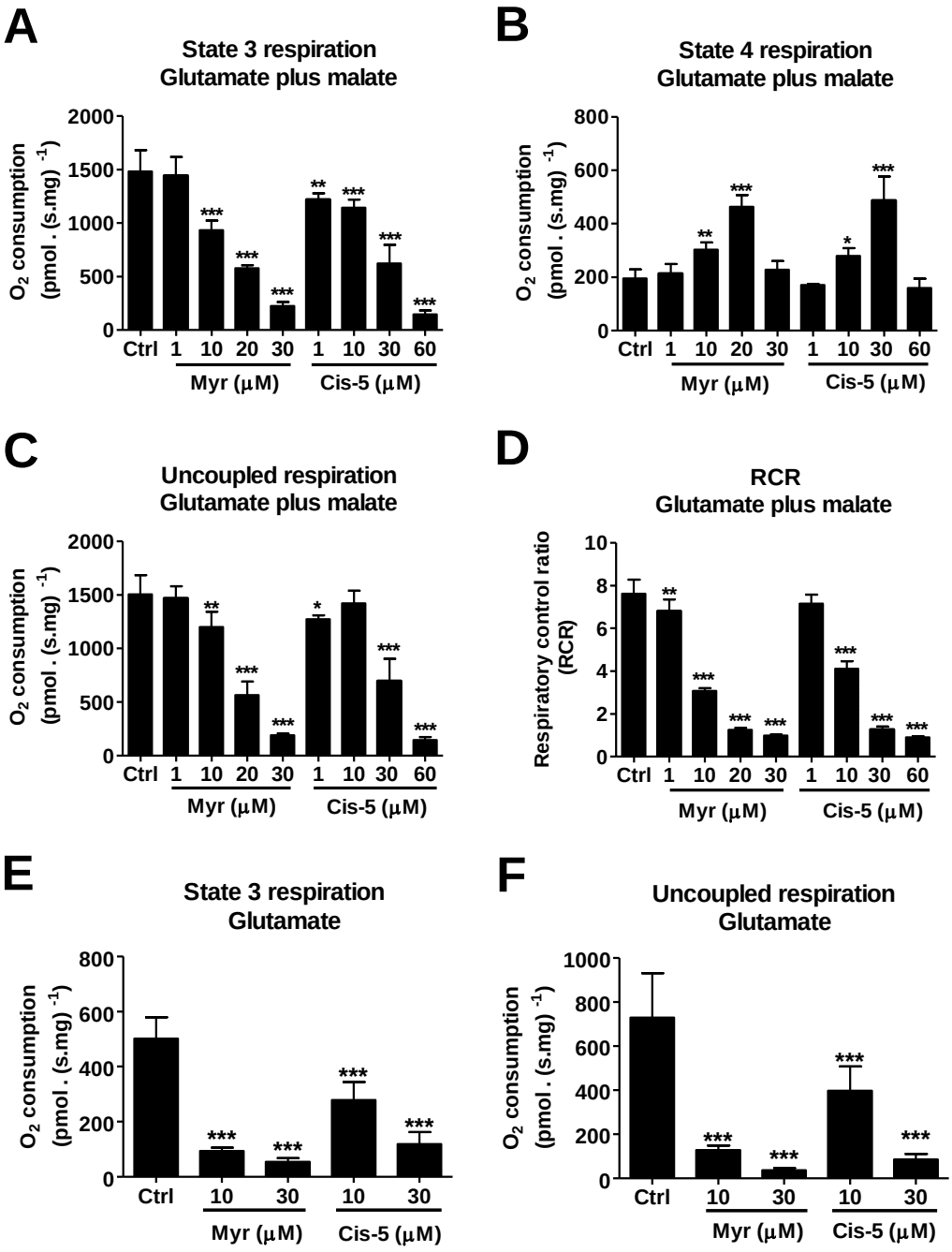

Fig 1. Effects of Myr and Cis-5 acids on respiratory parameters measured by oxygen consumption in glutamate plus malate or glutamate-supported skeletal muscle mitochondria. (A and E) State 3 (ADP-stimulated), (B) state 4 (resting), (C and F) uncoupled (CCCP-stimulated) respiration, (D) respiratory control ratio (RCR). Glutamate plus malate (2.5 mM each) (A-D) or glutamate (5 mM) (E and F) were used as substrates. Mitochondrial preparations (0.1 mg protein. mL^{-1}) and Cis-5 or Myr (1-60 μM, as indicated) were added to the incubation medium in the beginning of the assays. Controls were performed in the

absence of fatty acids. Values are means ± standard deviation of four to ten independent experiments (N) and are expressed as pmol O_2. s^{-1}. mg of protein^{-1}. *P < 0.05, **P < 0.01, ***P < 0.001, compared to controls (Duncan multiple range test).

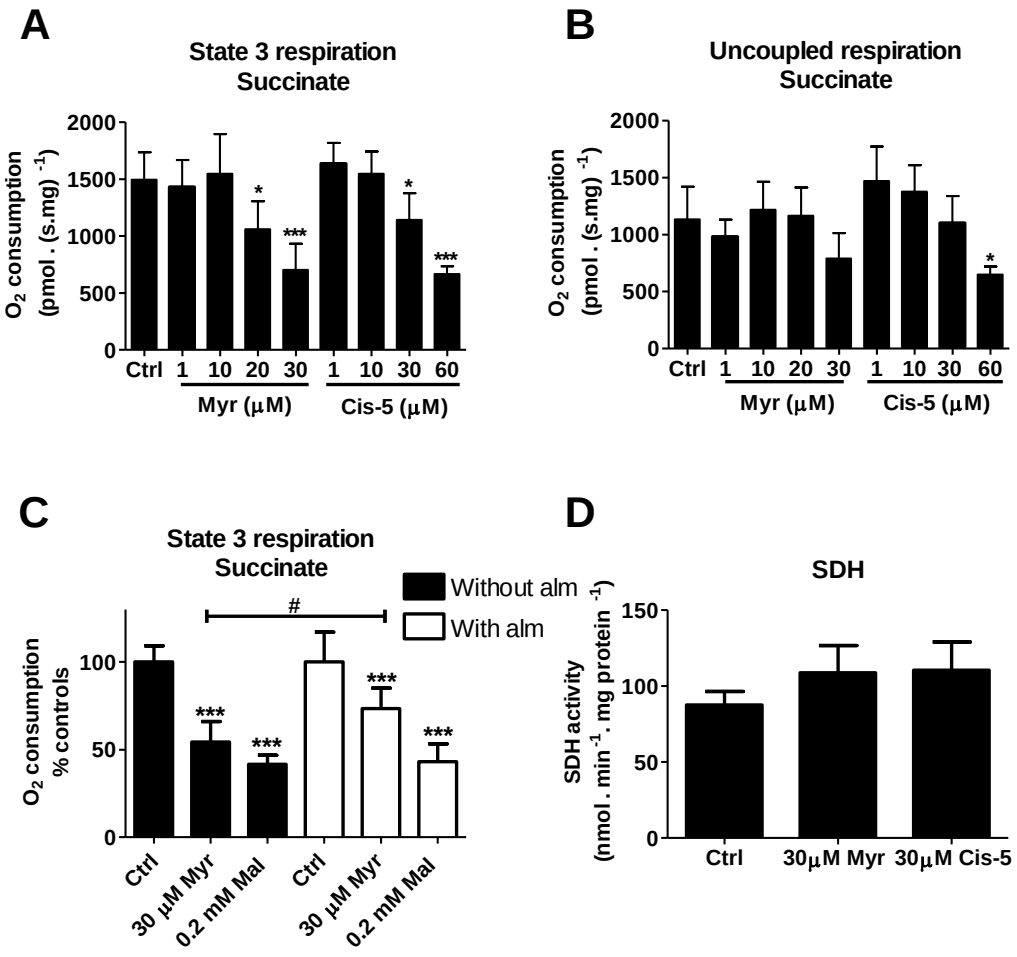

Fig 2. Effects of Myr and Cis-5 on respiratory parameters measured by oxygen consumption in succinate-supported skeletal muscle mitochondria and succinate dehydrogenase (SDH) activity. (A and C) State 3 (ADP-stimulated), (B) uncoupled (CCCP-stimulated) respiration and (D) SDH activity. (A-C) Succinate plus rotenone (5 mM and 1 µM, respectively) were used as substrates, mitochondrial preparations (0.1 mg protein. mL^{-1}) and Cis-5 or Myr (1-60 µM) were added to the incubation medium in the beginning of the assays. (C) Alamethicin (Alm, 40 µg/mg protein) and malonate (0.2 mM) were used in some groups and also added in the beginning of the assays. (D) SDH activity. Controls

were performed in the absence of fatty acids. Values are means ± standard deviation of three to ten independent experiments (N) and are expressed as pmol O_2. s^{-1}. mg of protein^{-1}. *P < 0.05, ***P < 0.001, compared to controls, $^{\#}P$ < 0.05, compared to Myr without alamethicin (Duncan multiple range test).

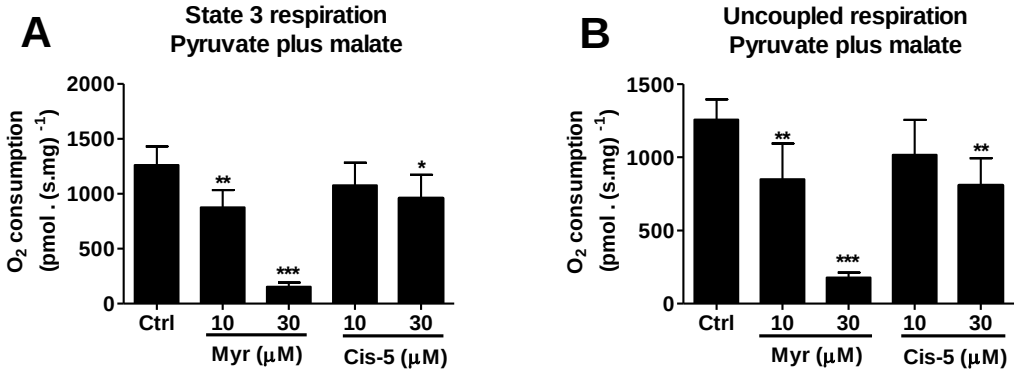

Fig 3. Effects of Myr and Cis-5 on respiratory parameters measured by oxygen consumption in pyruvate plus malate-supported skeletal muscle mitochondria. (A) State 3 (ADP-stimulated), (B) uncoupled (CCCP-stimulated) respiration. Pyruvate plus malate (2.5 mM each) were used as substrates. Mitochondrial preparations (0.1 mg protein. mL^{-1}) and Cis-5 or Myr (10 and 30 μM, as indicated) were added to the incubation medium in the beginning of the assays. Controls were performed in the absence of fatty acids. Values are means ± standard deviation of four to six independent experiments (N) and are expressed as pmol O_2. s^{-1}. mg of protein^{-1}. *P < 0.05, **P < 0.01, ***P < 0.001, compared to controls (Duncan multiple range test).

In order to evaluate the participation of reactive species generation in these alterations, we also assessed the effects of Myr on the respiratory parameters in glutamate plus malate-supported mitochondria using the antioxidants resveratrol (10 and 100 μM), coenzyme Q10 (50 and 100 μM) and creatine (1 mM). It was verified that these compounds were not capable to alter Myr-induced alterations on the respiratory parameters, making unlikely the involvement of oxidative attack

(Fig 4) [4A: $F_{(7,21)} = 12.34$, $P<0.001$; 4B: $F_{(7,21)} = 25.17$, $P<0.001$; 4C: $F_{(7,20)} = 6.66$, $P<0.001$; 4D: $F_{(7,21)} = 294.71$, $P<0.001$].

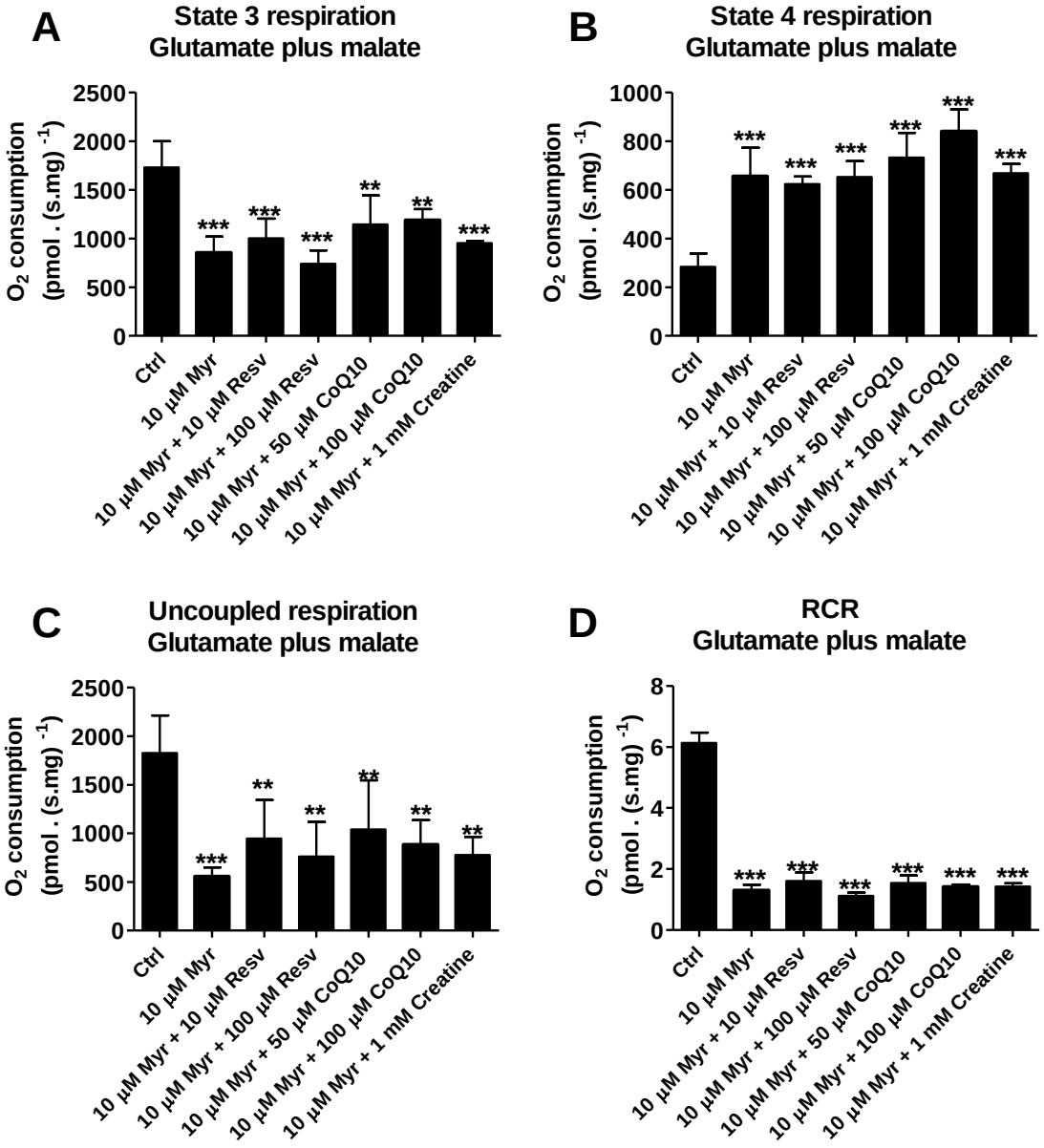

Fig 4. Antioxidants do not prevent Myr and Cis-5 acids alterations of respiratory parameters measured by oxygen consumption in glutamate plus malate-supported skeletal muscle mitochondria. (A) State 3 (ADP-stimulated), (B) state 4 (resting), (C) uncoupled (CCCP-stimulated) respiration, (D) respiratory control ratio (RCR). Glutamate plus malate (2.5 mM each) were used as substrates. Mitochondrial preparations (0.1 mg protein. mL^{-1}) and Myr (10 µM) were added to the incubation medium in the beginning of the assays, and co-

incubated with resveratrol (Resv, 10 and 100 µM), coenzyme Q10 (CoQ10, 50 and 100 µM) or creatine (1 mM) in some experiments. Controls were performed in the absence of fatty acids. Values are means ± standard deviation of three to five independent experiments (N) and are expressed as pmol O_2. s^{-1}. mg of $protein^{-1}$. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test).

3.2 Myristic acid (Myr) inhibits respiratory chain complex I-III activity in skeletal muscle mitochondria

In the next set of experiments we tested the effects of Myr (30 µM) on the respiratory chain complexes activities I-III, II and IV. Myr (30 µM) inhibited by about 70% complex I-III activity, without changing complexes II and IV activities (Fig 5) [5A: $F_{(2,13)} = 11.16$, $P<0.01$].

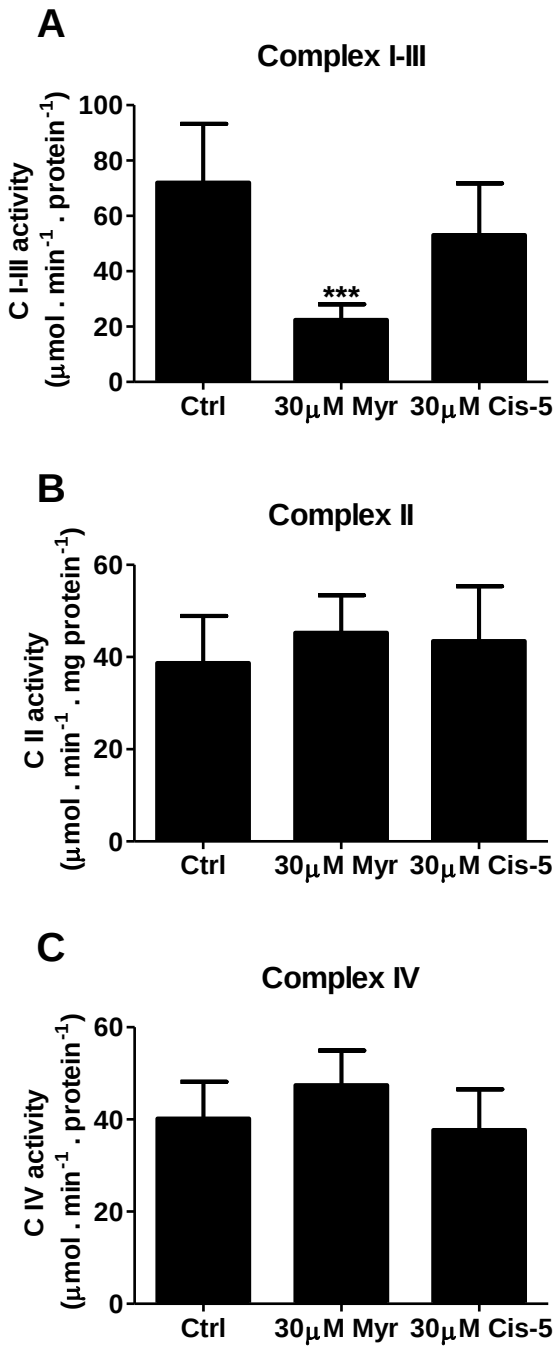

Fig 5. Effects of Myr and Cis-5 on the activity of respiratory chain complexes I-III (A), II (B) and IV (C) in skeletal muscle mitochondria. Mitochondrial preparations were pre-incubated for 30 min with Cis-5 (30 µM) or Myr (30 µM). Values are mean ± standard deviation of four to six independent experiments (animals) performed in triplicate and expressed as µmol cytochrome c reduced . min^{-1}. mg $protein^{-1}$ (A), µmol DCIP reduced . min^{-1}. mg $protein^{-1}$ (B) and µmol

cytochrome c oxidized . min^{-1}. mg $protein^{-1}$ (C). ***$P < 0.001$, compared to controls (Duncan multiple range test).

3.3 Myristic (Myr) and Cis-5-tetradecenoic (Cis-5) acids impair oxygen consumption in an integrated skeletal muscle cell system

We then investigated the influence of Myr and Cis-5 (30 µM) on respiratory parameters in permeabilized skeletal muscle fibers that represent a more physiological cellular milieu, using glutamate plus malate as substrates. Similar effects as those observed in isolated mitochondrial preparations were observed using this intact cell system (Fig. 6) [6A: $F_{(2,17)} = 5.18$, $P<0.05$; 6B: $F_{(2,19)} = 10.80$, $P<0.01$; 6C: $F_{(2,17)} = 42.12$, $P<0.001$; 6D: $F_{(2,16)} = 19.06$, $P<0.001$], validating therefore the effects observed in isolated mitochondria. Taken together, our results strongly support that Myr and Cis-5 significantly impair mitochondrial respiration by decreasing ADP-stimulated and uncoupled respiration, and increasing resting respiration.

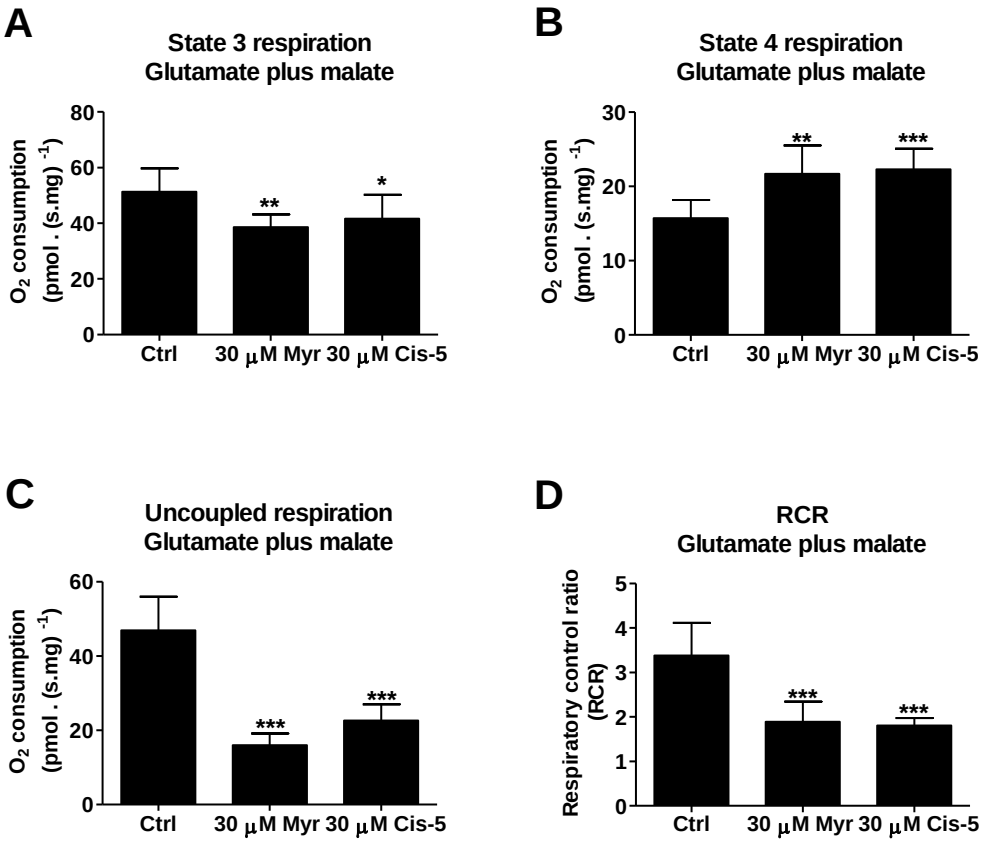

Fig 6. Effects of Myr and Cis-5 on respiratory parameters measured by oxygen consumption in permeabilized skeletal muscle fibers. (A) State 3 (ADP-stimulated), (B) state 4 (resting), (C) uncoupled (CCCP-stimulated) respiration, (D) respiratory control ratio (RCR). Glutamate (10 mM) and malate (5 mM) were used as substrates. Approximately 7 mg of plantar muscle and Myr or Cis-5 (30 µM) were added to 2 mL of the incubation medium in the beginning of the assays. Controls were performed in the absence of fatty acids. Values are means ± standard deviation of five to eight independent experiments (N) and are expressed as pmol O_2. s^{-1}. mg of $tissue^{-1}$. *P < 0.05, **P < 0.01, ***P < 0.001, compared to controls (Duncan multiple range test).

3.4 Myristic (Myr) and Cis-5-tetradecenoic (Cis-5) acids decrease ATP production in skeletal muscle mitochondria

Since alterations of the respiratory parameters may result in bioenergetics impairment associated with energy deprivation, the influence of Myr and Cis-5 on

ATP synthesis in mitochondria respiring with glutamate plus malate was determined. We found that Myr (10 and 30 µM) and Cis-5 (30 µM) significantly reduced the ATP production (Fig 7) [7: $F_{(4,25)}$ = 297.31, P<0.001], stressing that the effect caused by 30 µM Myr was similar to that provoked by the F_0-F_1 ATP synthase classical inhibitor oligomycin.

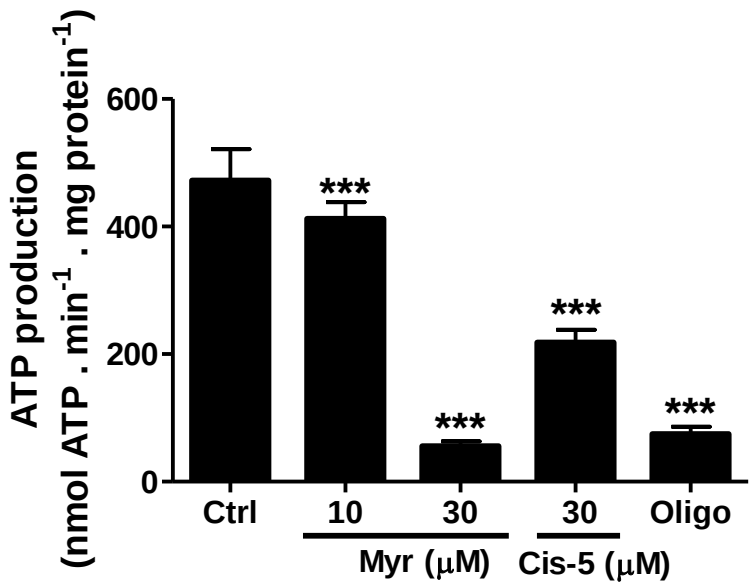

Fig 7. Effects of Myr and Cis-5 acids on ATP production in glutamate plus malate-supported skeletal muscle mitochondria. All experiments were performed in a reaction medium containing mitochondrial preparations (0.1 mg protein. mL-1) supported by glutamate plus malate (2.5 mM each). Myr (10 and 30 µM) or Cis-5 (30 µM) were added in the beginning of the assay. Controls were performed in the absence of fatty acids. Oligomycin A (Oligo, 1 µg. mL^{-1}) was used as a positive control. Values are means ± standard deviation of six independent experiments (animals) and are expressed as nmol ATP. min^{-1}. mg of $protein^{-1}$. ***P < 0.001 compared to controls (Duncan multiple range test)

3.5 Myristic (Myr) and Cis-5-tetradecenoic (Cis-5) acids decrease ΔΨm and induce MPT pore opening in skeletal muscle mitochondria

We next evaluated the effects of Myr and Cis-5 on mitochondrial ΔΨm using glutamate plus malate as substrates. In the absence of Ca^{2+}, both fatty

acids decreased ΔΨm at pathological (10-30 μM) but not at physiological (1 μM) concentrations (Fig 8 A and B). It is also clear from the figure that Myr- and Cis-5-induced inhibitory effects on ΔΨm were much greater after Ca^{2+} addition (A and B) [8A: $F_{(4,20)}$ = 22.58, P<0.001; 8B: $F_{(4,20)}$ = 34.12, P<0.001]. Figure 8C shows that these effects were less pronounced when pyruvate plus malate were used as substrates [8C: $F_{(2,9)}$ = 7.63, P<0.05].

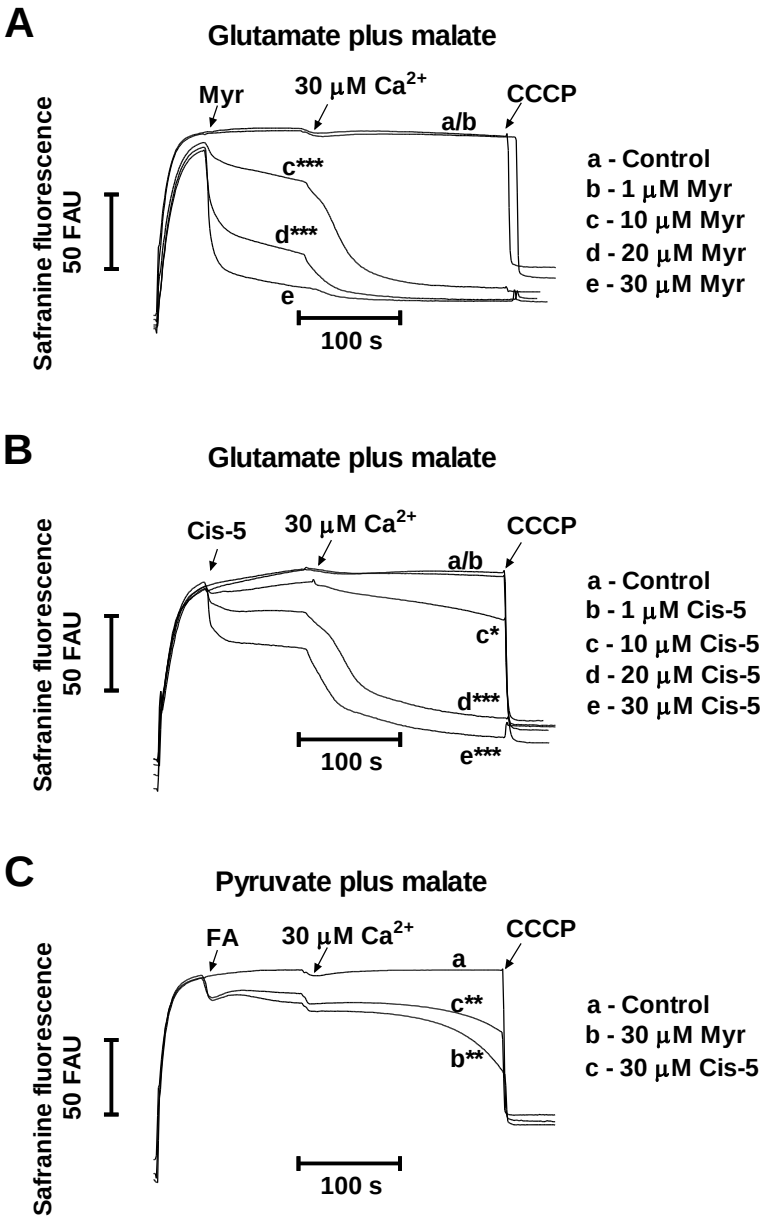

Fig 8. Effects of Myr and Cis-5 on mitochondrial membrane potential in skeletal muscle mitochondria. All experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein. mL^{-1}) supported by glutamate plus malate (2.5 mM each – panels A and B) or pyruvate plus malate (2.5 mM each – panel C). Myr (A - 1-30 μM, lines b-e; C – lines b and d) or Cis-5 (B - 1-30 μM, lines b-e; C – line c) were added 50 seconds after the beginning of the assay. All panels refer to mitochondrial preparations before and after addition of 30 μM Ca^{2+}, as indicated. Controls (lines a) were performed in the absence of fatty acids. CCCP was added at the end of the assays. Traces are representative of three to five independent experiments (N) and were expressed as fluorescence arbitrary units (FAU). *P < 0.05, **P < 0.01, ***P < 0.001 compared to controls (Duncan multiple range test).

Thereafter, we investigated whether Myr and Cis-5 were able to induce MPT pore opening by supplementing the medium with the MPT inhibitors CsA plus ADP in Ca^{2+}-supplemented mitochondria. It can be observed in figure 9 (A and B) [9A: $F_{(3,10)} = 23.09$, $P<0.001$; 9B: $F_{(3,9)} = 7.93$, $P<0.01$] that CsA plus ADP abolished ΔΨm decrease induced by Ca^{2+} plus Myr- and Cis-5and that RR, a mitochondrial calcium uniporter (MCU) inhibitor, was also able to prevent the ΔΨm decrease. The data strongly indicate the involvement of MPT and Ca^{2+} on ΔΨm dissipation elicited by these fatty acids in skeletal muscle mitochondria.

Since MPT pore opening may be a result of oxidative damage to the pore proteins, we tested whether the antioxidants melatonin or the thiol-alkylating agent (NEM) were able to prevent mitochondrial permeability transition and observed no alterations of Myr- and Cis-5-induced decrease of ΔΨm, making unlikely a role for oxidative stress in these effects. In contrast, the ANT inhibitor carboxyatractiloside (CAT) attenuated the uncoupling effect caused by Myr and Cis-5 observed before Ca^{2+} addition, indicating the involvement of this anion carrier in the ΔΨm

dissipation caused by Myr and Cis-5 (Fig. 9 C and D) [9C: $F_{(4,14)}$ = 76.67, P<0.001; 9D: $F_{(4,12)}$ = 142.60, P<0.001].

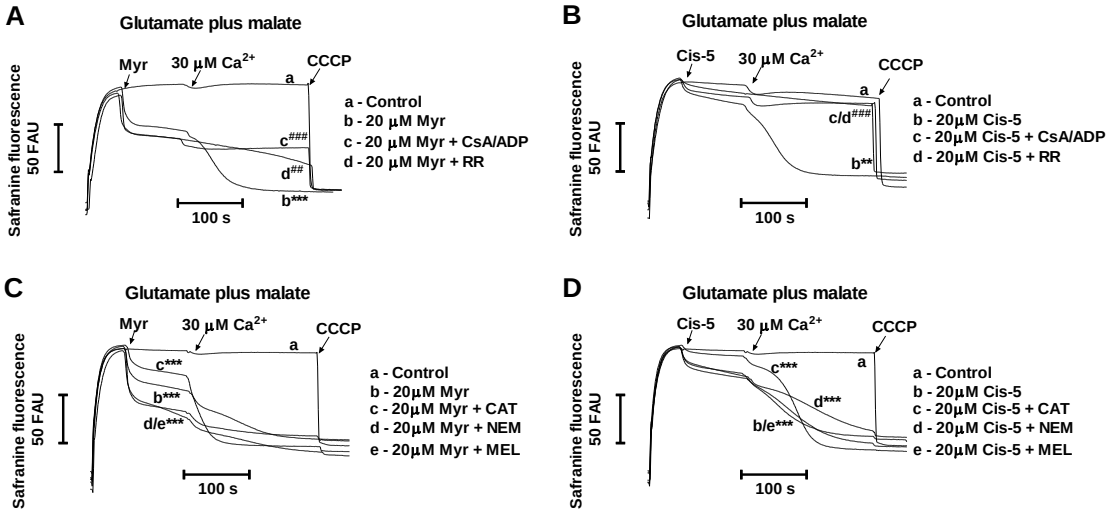

Fig 9. Effects of Myr and Cis-5 on mitochondrial membrane potential in skeletal muscle mitochondria. All experiments were performed in a reaction medium containing mitochondrial preparations (0.35 mg protein. mL^{-1}) supported by glutamate plus malate (2.5 mM each). (A and C) Myr (20 µM, lines b-e) or (B and D) Cis-5 (20 µM, lines b-e) were added 50 seconds after the beginning of the assay. (C) Cis-5 (30 µM, lines b-d). (D) Myr (20 µM, lines b-d). Cyclosporin A (CsA, 1 µM) plus ADP (300 µM), ruthenium red (RR, 1 µM), carboxyatractiloside (CAT, 5 µM), N-ethyl-maleymide (NEM, 10 µM) or melatonin (MEL, 1 mM) were added in the beginning of the assays and shown as indicated. All panels refer to mitochondrial preparations before and after addition of 30 µM Ca^{2+}, as indicated. Controls (lines a) were performed in the absence of fatty acids. CCCP was added at the end of the assays. Traces are representative of three to five independent experiments (N) and were expressed as fluorescence arbitrary units (FAU). **P < 0.01, ***P < 0.001 compared to controls, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, compared to Cis-5 or Myr (Duncan multiple range test).

3.5 Myristic (Myr) and Cis-5-tetradecenoic (Cis-5) acids decrease Ca^{2+} retention capacity in skeletal muscle mitochondria

Because MPT induction may compromise mitochondrial Ca^{2+} homeostasis potentially leading to mitochondrial release of this cation, we determined mitochondrial Ca^{2+} retention capacity in the presence of Myr and Cis-5 (10 and 30 μM) using glutamate plus malate as substrates. It can be seen in Fig. 10 that both fatty acids markedly reduced mitochondrial Ca^{2+} retention capacity (Fig. 10A) that was partially (30 μM Cis-5 or Myr) or totally (10 μM Cis-5 or Myr) prevented by CsA, reinforcing the induction of MPT pore opening by Myr and Cis-5.

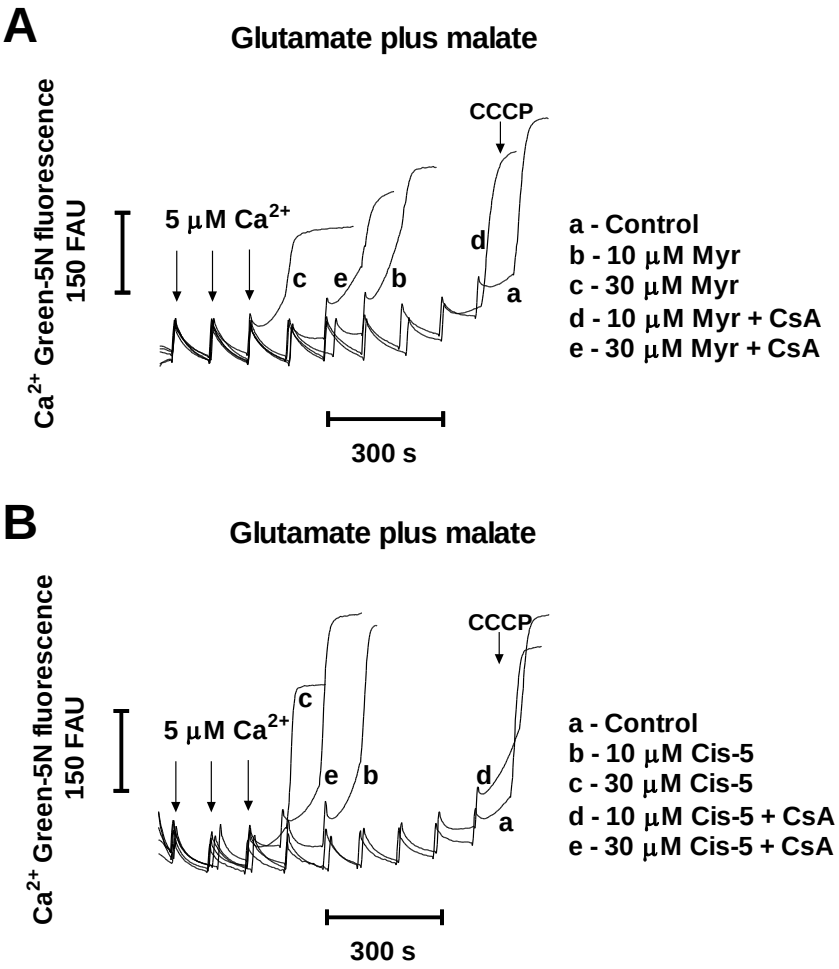

Fig 10. **Effects of Myr and Cis-5 on mitochondrial Ca^{2+} retention capacity in Ca^{2+}-loaded skeletal muscle mitochondria.** All experiments were performed in

a reaction medium containing mitochondrial preparations (0.35 mg protein. mL^{-1}) supported by glutamate plus malate (2.5 mM each). The fatty acids were added in the beginning of the assays. (A) Myr (10 and 30 µM, lines b-e). (B) Cis-5 (10 and 30 µM, lines b-e). Cyclosporin A (CsA, 1 µM, line d and e) was added in the beginning of the assay. Mitochondrial preparations were supplemented by successive additions of 5 µM Ca^{2+} every 2 min, as indicated by the arrows. Controls (lines a) were performed in the absence of these fatty acids. CCCP was added at the end of the assays. Traces are representative of three independent experiments (N) and were expressed as fluorescence arbitrary units (FAU).

4. Discussion

The late-onset myopathic form is the most common variant of VLCAD deficiency manifesting with skeletal myopathy and rhabdomyolysis, especially during intense physical exercise or catabolic events [8, 40, 41]. During episodes of rhabdomyolysis there is accumulation of long-chain acylcarnitines (up to 200 nmol/g ~ 200 µM) and elevation of creatine kinase activity up to 60,000 U/L suggesting massive muscle cell destruction [42]. These observations indicate that myopathy of VLCAD deficient patients may be attributed to the accumulating metabolites, including long-chain acylcarnitines and/or their free fatty acid derivatives. However, limited information is available on the underlying pathogenetic mechanisms of tissue damage and to the best of our knowledge no report exist on the pathogenesis of the myopathic form of this disorder despite that VLCAD deficiency was described over 25 years ago [43]. In this context, mild lipidosis and subsarcolemmal mitochondrial accumulation associated with a combined defect of complexes I + III and II + III and reduced level of coenzyme Q was previously described in muscle biopsies from affected patients [23] that may be tentatively attributed to the toxic effects of the accumulating metabolites. Furthermore, the observations of increased levels of long-chain acylcarnitines,

particularly tetradecenoylcarnitine (C14:1), as well as of cis-5-tetradecenoic (C14:1, Cis-5) and myristic (C14, Myr) acids suggest that these metabolites may be involved with the muscular damage [44]. Therefore, this investigation aimed to investigate the role of Cis-5 and Myr, the major fatty acids that accumulate in VLCAD deficiency, on critical mitochondrial functions in skeletal muscle of adolescent rats that is highly dependent on energy production.

We first observed that Cis-5 and Myr provoked a marked decrease of ADP-stimulated and uncoupled respiration, which are characteristic properties of metabolic inhibitors. Furthermore, these inhibitory effects were more pronounced when glutamate plus malate or pyruvate plus malate were used as respiratory substrates, as compared to succinate, implying that NADH-linked respiration was more vulnerable to the fatty acids-elicited toxic effects. These findings could be secondary to inhibition of complex I since Myr significantly inhibited complexes I-III activity of the respiratory chain. On the other hand, Myr-induced decrease of ADP-stimulated succinate-supported respiration was attenuated in alamethicin-permeabilized mitochondria suggesting a competition between succinate and Myr for the same transporter, which was previously demonstrated to occur in the liver [45]. Furthermore, Cis-5 and Myr did not change SDH activity, ruling out an inhibitory effect on this enzyme to explain the decreased oxygen consumption of succinate-linked respiration. It was also observed that the antioxidants resveratrol, coenzyme Q10 and creatine were not able to prevent Myr-induced alterations of the respiratory parameters, making unlikely the involvement of oxidative stress in these effects. The degree of impairment of mitochondrial bioenergetics by these fatty acids was confirmed by the findings showing a marked reduction of ATP synthesis.

It was also observed that Cis-5 and Myr significantly increased resting respiration and decreased ΔΨm that are usually caused by uncouplers of oxidative phosphorylation, suggesting an uncoupling effect of these compounds. The ANT inhibitor carboxyatractiloside (CAT) attenuated Myr- and Cis-5- induced ΔΨm dissipation suggesting the participation of this anion carrier in this uncoupling effect. In this particular, the protonophoric action of Cis-5 and Myr may be due to the transbilayer movement of their undissociated forms into the mitochondrial matrix and by the passage of the dissociated forms of these fatty acids in the opposite direction to the intermembrane mitochondrial space possibly by ANT [46, 47]. However, we cannot rule out the possibility that the uncoupling behavior of Cis-5 and Myr could be also associated with other mitochondrial carriers, including the UCP [48, 49], glutamate/aspartate antiporter [50, 51], the mono- and tri-carboxylate carriers [52] and the phosphate carrier [53].

Other important findings of the present investigation were that Myr and Cis-5 significantly decreased ADP-activated respiration, uncoupled respiration and RCR, and increased resting respiration in permeabilized skeletal muscle fibers that consist of an integrated cell system, supporting therefore the data obtained in isolated mitochondria.

It was also verified that ΔΨm decrease was accentuated after Ca^{2+} addition to the mitochondrial fractions and that the combination of the inhibitors of MPT CsA plus ADP, as well RR, a MCU inhibitor, totally prevented Myr- and Cis-5-induced ΔΨm dissipation. The data strongly indicate the involvement of MPT and a role for Ca^{2+} on ΔΨm dissipation elicited by these fatty acids in Ca^{2+}-loaded skeletal muscle mitochondria. Moreover, it is unlikely that MPT pore opening caused by these fatty acids could be due to oxidative damage to the pore proteins

since the antioxidants melatonin and the thiol-alkylating agent (NEM) were unable to prevent MPT induction.

Another important contribution of the present study was that Cis-5 and Myr markedly disrupted the mitochondrial capacity to uptake and retain Ca^{2+}, which is critical to cell Ca^{2+} homeostasis [54-58]. Moreover, this effect was totally prevented by CsA, indicating that the reduction of mitochondrial Ca^{2+} retention capacity was a consequence of MPT induction. In this particular, it is feasible that MPT pore opening could allow Ca^{2+} release from the matrix, overcoming mitochondrial Ca^{2+} retention capacity [59-61]. It should be also considered that MPT induction results in mitochondrial depolarization, organelle swelling, loss of metabolites (Ca^{2+}, Mg^{2+}, glutathione, NADH, and NADPH), and release of mitochondrial proapoptotic factors leading to apoptosis and necrosis, as well as impairment of oxidative phosphorylation and ATP synthesis [59-65].

The data shown in the present work are in accordance with previous findings obtained in liver demonstrating that Myr causes bioenergetics dysfunction and induces MPT in rat liver [66-68]. However, to the best of our knowledge there is no study performed in skeletal muscle on the toxic effects of Myr and also of Cis-5, which is the biochemical hallmark of VLCAD deficiency. However, a previous study from our lab demonstrated that Myr and Cis-5 disrupt mitochondrial bioenergetics and Ca^{2+} homeostasis, behaving as uncouplers, metabolic inhibitors and inductors of MPT in rat heart [28], implying similar mechanisms caused by these fatty acids on muscular tissues.

At the present, it is difficult to establish the pathophysiological significance of our data since the levels of Cis-5 and Myr in skeletal muscle of patients affected by VLCAD deficiency are so far unknown. However, it is stressed that

these fatty acids disturbed crucial mitochondrial functions in the skeletal muscle at concentrations similar to those found in plasma of the affected patients [44]. Since rhabdomyolysis is usually caused by ATP depletion [69] and may also be associated with imbalance of intracellular calcium homeostasis [70] and MPT [71], it is possible that our present data may contribute to explain the rhabdomyolysis and muscular symptoms, as well as the highly increased levels of CK during exercise reflecting muscular damage.

5. Conclusions

In conclusion, we provide for the first time experimental evidence that Cis-5 and Myr, which most accumulate in VLCAD deficiency, deregulate mitochondrial functions, behaving as strong metabolic inhibitors, uncouplers of oxidative phosphorylation and MPT inductors in skeletal muscle from young rats. We postulate that disruption of mitochondrial homeostasis caused by Cis-5 and Myr may be related to the myopathy associated with muscular weakness, pain and rhabdomyolysis observed in patients affected by VLCAD deficiency especially during strong exercise that precipitates this symptomatology and when plenty energy should be available for the muscle.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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PARTE III: Discussão e Conclusões

3. DISCUSSÃO

As deficiências hereditárias da oxidação de ácidos graxos são doenças associadas com alta morbidade e mortalidade, e por isso tem atraído muito interesse nos últimos anos, sendo algumas delas incluídas na triagem neonatal em massa em muitos países. O diagnóstico dessas doenças no recém-nascido demonstrou que a incidência dessas doenças é mais alta do que originalmente estabelecido em triagens seletivas (pacientes sintomáticos). A implementação da triagem neonatal permite que logo após o diagnóstico pré-sintomático os pacientes recebam tratamento imediatamente após o nascimento, impedindo ou diminuindo os efeitos deletérios agudos e crônicos encontrados em pacientes afetados por essas doenças (MERRITT; NORRIS; KANUNGO, 2018; WILCKEN et al., 2003). No entanto, o tratamento desses distúrbios metabólicos por restrição dietética e suplementação com MCT como é feito atualmente não consegue prevenir por completo a morbidade e também a mortalidade, se fazendo, portanto, necessário esclarecer os complexos mecanismos patogênicos na VLCADD.

A obtenção de energia a partir de ácidos graxos de cadeia longa (LCFA) está muito prejudicada na VLCADD. Assim, a sintomatologia piora durante episódios catabólicos da doença como o jejum ou os processos infecciosos, afetando os tecidos que mais utilizam LCFA como fonte de energia primária, tais como o coração, fígado e músculo esquelético (MC et al., 2004). Pacientes com VLCADD geralmente apresentam manifestações cardíacas severas, nas quais a privação de energia parece desempenhar um papel importante (MATHUR et al., 1999). Entretanto, os mecanismos precisos envolvendo as manifestações clínicas estão ainda pouco estabelecidos. Como dito anteriormente, durante as crises agudas de descompensação metabólica, os sintomas e sinais clínicos se acentuam,

principalmente com arritmia cardíaca e insuficiência hepática, que coincidem com um aumento acentuado nos tecidos e nos líquidos biológicos das concentrações dos ácidos graxos e de seus derivados de carnitina, tornando concebível que esses compostos acumulados possam contribuir para a patogênese dessa doença.

A mortalidade e a morbidade na maioria das crianças com VLCADD são diminuídas pelo diagnóstico pela triagem neonatal expandida e o tratamento precoce, com restrição de ácidos graxos de cadeia longa (LCFA), sugerindo que esses compostos possam contribuir para o dano tecidual e os sintomas apresentados pelos pacientes (SPIEKERKOETTER, 2010; SPIEKERKOETTER; LINDNER; SANTER; GROTZKE; BAUMGARTNER; BOEHLES; DAS; HAASE; HENNERMANN; KARALL; KLERK; et al., 2009; SPIEKERKOETTER et al., 2003; WANDERS et al., 1999). Além disso, os episódios com manifestações cardíacas severas não podem ser completamente prevenidos, indicando que a terapia atual não é bem sucedida, e enfatizando a necessidade de se investigar os mecanismos de patogênese dessa doença para o desenvolvimento de outras estratégias terapêuticas (WAISBREN et al., 2013).

As alterações morfológicas mitocondriais, rabdomiólise e acidemia lática que foram reportadas em alguns pacientes afetados (DIEKMAN et al., 2014; ENGBERS et al., 2005; SAUDUBRAY et al., 1999; VENTURA et al., 1998), bem como esteatose hepática, proliferação mitocondrial no coração (LAFORET et al., 2009; PRIMASSIN et al., 2010; TUCCI et al., 2010), e dano na bioenergética mitocondrial em tecidos obtidos a partir de um modelo genético animal com VLCADD (EXIL et al., 2006; TUCCI et al., 2014) indicam que um comprometimento da homeostase energética mitocondrial pode estar envolvido na patogênese dessa doença. Neste particular, estudos prévios demonstraram que os derivados de acil-

CoA de cadeia longa inibem a síntese de ATP e o consumo de oxigênio (estado 3) em mitocôndrias de fígado (VENTURA et al., 2005). Além disso, o Myr demonstrou ser capaz de desacoplar a OXPHOS com envolvimento do carreador de dicarboxilatos (WIECKOWSKI; WOJTCZAK, 1997) e do translocador de nucletídeos de adenina (ANT) (BODROVA et al., 2000), bem como induzir a abertura do PTP sensível à ciclosporina A (CsA), inibidora da CyP D, em mitocôndrias de fígado carregadas com Ca^{2+} (BODROVA et al., 2003; WIĘCKOWSKI; WOJTCZAK, 1998).

No entanto, não há na literatura estudos sobre os efeitos do Myr em outros tecidos e especialmente do Cis-5, que é o marcador bioquímico para o diagnóstico da VLCADD (ROE, CHARLES R; DING, 2005), sobre funções mitocondriais críticas e a suscetibilidade dos diversos tecidos afetados. Portanto, nesse trabalho estudamos os efeitos do Cis-5 e Myr sobre uma ampla gama de importantes parâmetros bioenergéticos em preparações mitocondriais de coração, fígado e músculo esquelético, bem como em células cultivadas e fibras musculares, na esperança de esclarecer os mecanismos pelos quais esses ácidos graxos prejudicam a homeostase celular.

De uma forma geral, demonstramos que o Cis-5 e o Myr, em concentrações patológicas (10 – 60 µM) (COSTA et al., 1998), mas não fisiológicas (1 – 5 µM), comprometem a bioenergética mitocondrial, atuando como desacopladores, inibidores metabólicos e indutores da abertura do PTP, causando a diminuição da produção de ATP, além de diminuir a capacidade de retenção de Ca^{2+} em mitocôndrias isoladas de coração, fígado e músculo esquelético, bem como em fibras musculares cardíacas e esqueléticas de ratos e em células hepáticas e cardíacas cultivadas.

Inicialmente, verificamos que o Cis-5 e o Myr, em concentrações similares às observadas no plasma de pacientes, aumentaram o consumo de oxigênio no estado de repouso (estado 4, após adição de oligomicina) da respiração mitocondrial e diminuíram o RCR em preparações mitocondriais obtidas de fígado, músculo cardíaco e esquelético, com todos substratos testados, indicando um comportamento desacoplador. Por outro lado, ambos ácidos graxos diminuíram marcadamente e de forma dose-dependente o consumo de oxigênio no estado 3 (estimulado por ADP) e desacoplado (após adição de CCCP) da respiração mitocondrial, utilizando glutamato mais malato como substrato, principalmente em mitocôndrias de músculo cardíaco e esquelético, com menor efeito em fígado, corroborando com a hipótese de que esses metabólitos também agem como potentes inibidores metabólicos em mitocôndrias. Também utilizamos somente glutamato ou piruvato mais malato como substratos e os efeitos provocados pelos Cis-5 e Myr foram semelhantes.

No entanto, quando utilizamos sucinato como substrato os efeitos no consumo de oxigênio foram menos pronunciados do que aqueles observados utilizando-se glutamato mais malato em todos os tecidos testados, sugerindo que a possibilidade de que esses ácidos graxos prejudicam o transporte ou a oxidação de glutamato ou piruvato. Esta pode ocorrer pela inibição da atividade do complexo I, ou pela redução da atividade da AST que é necessária para a oxidação do glutamato, ou ainda devido a um bloqueio enzimático do ciclo do ácido cítrico. De fato, observamos inibição na atividade do complexo I da cadeia respiratória em mitocôndrias de coração e do complexo I-III em mitocôndrias de fígado e músculo esquelético, sem alterações nos complexos II ou IV. Nesse contexto, já foi observado inibição da atividade do complexo I da cadeia respiratória, principalmente,

mas também do II e III, associadas ao acúmulo dos ácidos graxos em alguns pacientes com deficiência da LCHAD (TYNI et al., 1996). Cis-5 e Myr também inibiram seletivamente a atividade da α-KGDH do ciclo do ácido cítrico em mitocôndrias de fígado, sem alterar as atividades da GDH, SDH, MDH, IDH, CS deste ciclo, bem como a AST.

Por outro lado, sabe-se que o transporte de glutamato para dentro da mitocôndria é dependente do potencial de membrana formado, uma vez que ao entrar uma molécula de glutamato também entra um próton, e por isso esse transporte pode ser prejudicado por desacoplamento (GNAIGER, 2014). No que se refere ao menor efeito observado sobre a respiração celular quando do uso de sucinato como substrato, observamos que o transporte de sucinato foi prejudicado pelo Myr em mitocôndrias de músculo esquelético e cardíaco, uma vez que a presença da alameticina, que forma poros na membrana mitocondrial e, portanto, permite o livre acesso do sucinato ao interior da mitocôndria, atenuou os efeitos inibitórios causados no estado 3 da respiração, sugerindo uma competição entre o Myr e o sucinato pelo carreador de dicarboxilatos. Resultados semelhantes foram previamente demonstrados em fígado de ratos (WIECKOWSKI; WOJTCZAK, 1997).

Como mencionado acima, o aumento da respiração de repouso (estado 4) causado pelo Cis-5 e Myr e a diminuição o RCR, aliados com a dissipação do $\Delta\Psi m$ não dependente de Ca^{2+} são indicativos de efeito desacoplador da OXPHOS por parte desses ácidos. Efeitos similares tinham sido obtidos com o Myr em estudos prévios no fígado e no coração (SCHONFELD et al., 1996; WOJTCZAK; WIECKOWSKI; SCHONFELD, 1998). O ANT provavelmente está envolvido nesse efeito desacoplador, uma vez que o carboxiatractilosídeo (CAT), inibidor do ANT, atenuou o aumento do estado 4 em coração e fígado, bem como a dissipação do

ΔΨm causados por Cis-5 e Myr em mitocôndrias de músculo esquelético e cardíaco. Resultados prévios demonstraram que o CAT preveniu a dissipação do ΔΨm causada por Myr em mitocôndrias de fígado de ratos adultos, reforçando, portanto, a importância do ANT nesses efeitos (SCHONFELD; BOHNENSACK, 1997; WIĘCKOWSKI; WOJTCZAK, 1998). Em contraste, proteínas desacopladoras (UCP) e o antiporter glutamato/aspartato não estão envolvidos no desacoplamento, pois verificamos que seus respectivos inibidores, guanosina difosfato (GDP) e dietil pirocarbonato (DPC), não alteraram significantemente esse efeito (AFFOURTIT et al., 2007; MOKHOVA; KHAILOVA, 2005; SKULACHEV, VLADIMIR P., 1998). Acreditamos que o efeito desacoplador da OXPHOS pode ser resultado de um mecanismo protonofórico com um movimento transmembrana dos ácidos Cis-5 e Myr não-dissociados através da membrana mitocondrial interna, sendo facilitado pelo ANT (GOGLIA; SKULACHEV, 2003; SCHONFELD, 1992; SCHONFELD; SCHILD; KUNZ, 1989), o que vai ao encontro dos resultados prévios dos efeitos do Myr (BODROVA et al., 2000; SAMARTSEV; MARCHIK; SHAMAGULOVA, 2011). Considerando que a atividade do ANT e do estado 3 são fundamentais para a produção de ATP, sugere-se que a interação desses ácidos graxos com o ANT pode estar envolvida nos seus efeitos deletérios, comprometendo a bioenergética nos músculos cardíaco e esquelético, e no fígado. Por outro lado, já que o CAT atenuou, mas não preveniu completamente o desacoplamento do Cis-5 e Myr, não podemos excluir a possibilidade dos ânions de LCFA serem transferidos por outros carreadores mitocondriais, como os carreadores de mono e tricarboxilatos (SCHONFELD; WIECKOWSKI; WOJTCZAK, 2000), bem como pelo carreador de fosfato (ZACKOVA; KRAMER; JEZEK, 2000).

Também demonstramos que os Cis-5 e Myr diminuíram a produção de ATP em mitocôndrias de fígado, coração e músculo esquelético, refletindo alterações bioenergéticas severas nesses tecidos, reforçando uma inibição metabólica importante causada por esses LCFA, provavelmente levando a célula a um déficit energético.

No presente trabalho também foi mostrado que o Cis-5 e o Myr dissiparam o ΔΨm mitocondrial na ausência de Ca^{2+} em todos os tecidos testados, que também pode ser atribuído a um efeito desacoplador ou alternativamente a uma inibição metabólica ou aos dois mecanismos. Além disso, essa dissipação do ΔΨm foi maior e de forma dose dependente na presença de Ca^{2+}, utilizando glutamato mais malato como substratos. Com o intuito de verificar se os efeitos observados no ΔΨm foram atingidos apenas com o uso do glutamato mais malato como substratos, também testamos esse parâmetro utilizando outros substratos, como o sucinato em mitocôndrias de fígado e piruvato mais malato no músculo esquelético. Uma vez mais verificamos que os efeitos causados pelos LCFA são de maior magnitude com o glutamato mais malato, quando comparado ao efeitos observamos com sucinato ou piruvato mais malato, indicando que é mais provável que os Cis-5 e Myr estejam prejudicando de forma mais marcada as vias de oxidação do glutamato.

Em todos os tecidos testados, a dissipação do ΔΨm foi completamente prevenida por rutênio vermelho (RR), um inibidor do uniporte mitocondrial de Ca^{2+} (MOORE, 1971), bem como por CsA mais ADP (ROTTENBERG; MARBACH, 1989; SAITO; CASTILHO, 2010). Uma vez que a CsA inibe o PTP inativando a Cyp D, uma proteína da matriz associada com a formação do PTP (BASSO et al., 2005; POTTECHER et al., 2013; TANVEER et al., 1996; YARANA et al., 2012), enquanto que o ADP se liga ao ANT pelo lado da matriz mitocondrial inibindo o PTP

(ROTTENBERG; MARBACH, 1989; SAITO; CASTILHO, 2010), nossos resultados indicam que os Cis-5 e o Myr induzem a abertura do PTP.

A abertura do PTP pode ser provocada de diversas formas, tais como por estresse oxidativo, alterações na estrutura da membrana mitocondrial e dano ao DNA (NELSON; COX, 2017). Neste particular, é improvável que o dano às proteínas do poro tenha ocorrido por um ataque oxidativo por espécies reativas sobre tais proteínas, induzindo secundariamente a abertura do PTP causada por esses ácidos graxos, uma vez que eles não foram capazes de causar estresse oxidativo, medido pelos níveis de malondialdeído (MDA) e GSH, mesmo em altas concentrações (100 µM) no coração. Além disso, os efeitos deletérios causados pelo Myr observados na respiração em mitocôndrias de músculo esquelético não foram prevenidos pelos antioxidantes resveratrol, coenzima Q10 e creatina. Essa conclusão é reforçada pelo fato de que o ditiotreitol (DTT), potente agente redutor, também não ter sido capaz de prevenir a indução da abertura do PTP pelo Myr em coração, assim como o agente alquilante de tióis N-etilmaleimida (NEM) e o antioxidante melatonina que não foram capazes de prevenir a dissipação do ΔΨm em mitocôndrias de músculo esquelético, bem como os antioxidantes resveratrol e coenzima Q10 nas mitocôndrias de fígado.

Também é improvável que mudanças na fluidez da membrana mitocondrial possam estar associadas com a indução ao PTP (COLELL et al., 2003; RICCHELLI et al., 1999) explicando os achados da presente investigação, uma vez que a anisotropia não foi alterada por esses metabólitos em coração e em fígado.

Uma vez que a mitocôndria é responsável por captar cálcio intracelular em situações fisiológicas e patológicas, sendo importante no controle da homeostase celular de cálcio, avaliamos se o Cis-5 e o Myr poderiam alterar esse parâmetro.

Observamos que esses ácidos graxos foram capazes de diminuir significativamente em todos os tecidos testados a capacidade de retenção de Ca^{2+}, uma importante função mitocondrial (BAUGHMAN et al., 2011; DE STEFANI et al., 2011; MARCHI; PINTON, 2014; PAN et al., 2013; PENDIN et al., 2014), especialmente em tecidos musculares, constituindo um mecanismo regulatório muito importante para a fisiologia cardíaca necessária para o funcionamento dos cardiomiócitos e o mecanismo de contração do músculo esquelético (DRAGO et al., 2012; KWONG et al., 2018). É possível que a ativação do PTP possa estar envolvida na diminuição da capacidade de retenção de Ca^{2+}, uma vez que essa condição leva à perda de elementos mitocondriais (Ca^{2+}, Mg^{2+}, GSH, NADH e NADPH), resultando numa disfunção na OXPHOS (RASOLA; BERNARDI, 2011; STARKOV, 2010; ZORATTI; SZABO, 1995). Entretanto, não podemos excluir a possibilidade de que o prejuízo na capacidade de captar e reter Ca^{2+} induzido por Cis-5 e Myr também possa ser parcialmente devido ao efeito desacoplador desses ácidos graxos, levando subsequentemente a uma permeabilização não seletiva e liberação mitocondrial de Ca^{2+}.

Outras observações importantes do presente trabalho são que o Cis-5 e o Myr induziram inchamento mitocondrial e liberação de citocromo *c* em mitocôndrias de fígado carregadas de Ca^{2+}, provavelmente uma consequência da indução do PTP que está de acordo com o conceito que o colapso no $\Delta\Psi m$ causado pela ativação do PTP é comumente associado com inchamento, onde a membrana mitocondrial interna expande mais que a externa, levando a ruptura desta útilma e perda de fatores apoptóticos, como citocromo *c*, que podem potencialmente provocar apoptose e/ou necrose celular (BERNARDI, P., 1999; BERNARDI, PAOLO, 2013; SKULACHEV, V. P., 1996).

O conteúdo de NAD(P)H da matriz mitocondrial de coração também foi muito diminuído pelos Cis-5 e Myr na ausência de Ca^{2+}. Nesse particular, é pouco provável que essa redução ocorreu por oxidação dos equivalentes reduzidos causada por espécies reativas porque esses ácidos foram incapazes de causar estresse oxidativo no coração, levando-se então à possibilidade de que essa redução ocorreu devido ao efeito desacoplador desses ácidos graxos.

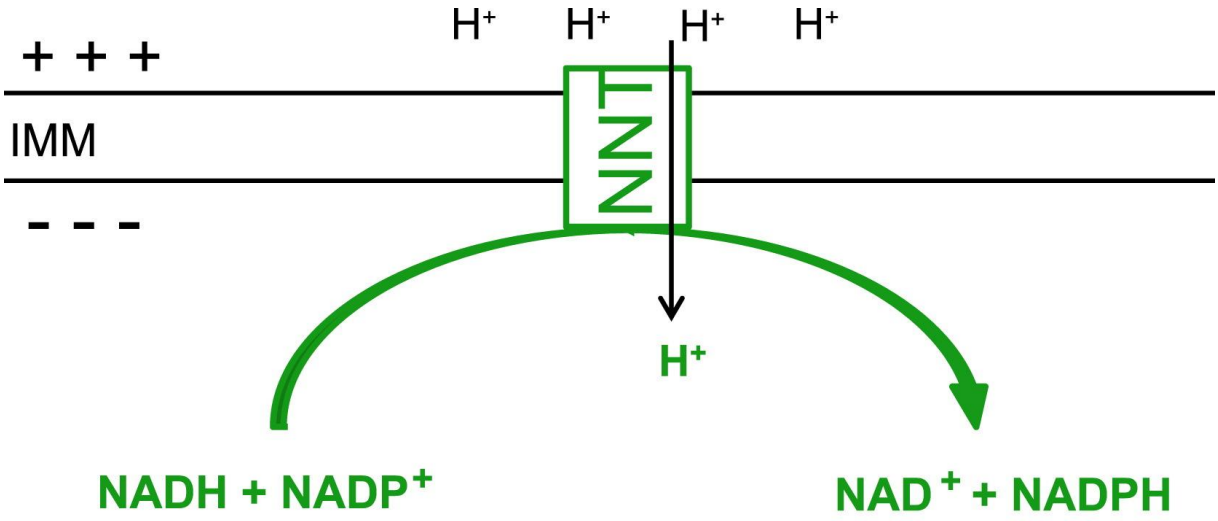

Figura 6. Esquema da reação catalizada pela transidrogenase mitocondrial de nucleotídeos de nicotinamida (NNT). NNT está embebida na memmbrana mitocondrial interna (IMM) e transloca H^+ para a matriz enquanto $NADP^+$ é reduzido com o gasto de NADH da matriz. O gradiente eletroquímico através da IMM desloca o equilíbrio da reação para a direita (adaptado de RONCHI et al. (2013)).

Enfatize-se que a inibição metabólica demonstrada no presente trabalho pela redução do estado 3 da respiração mitocondrial, bem como da síntese de ATP e do conteúdo de equivalentes reduzidos NAD(P)H mitocondrial pode provocar uma diminuição da razão NADH/NAD. Nessas condições, o sentido da reação da enzima transidrogenase mitocondrial de nucleotídeos de nicotinamida (NNT), que usualmente converte o NADH em NADPH, fica invertido (figura 6) (RYDSTROM, 2006). Na forma reversa da NNT, ocorre consumo de NADPH para formar NADH com o objetivo de manter o metabolismo energético, resultando na formação do produto oxidado NADP (NICKEL et al., 2015). Por sua vez, o NADPH é importante

na manutenção do estado redox e da defesa mitocondrial contra o estresse oxidativo por atuar como cofator e agente redutor na regeneração dos sistemas antioxidantes glutationa redutase/peroxidase e tioredoxina redutase/peroxiredoxina (NICKEL et al., 2015) (KOWALTOWSKI et al., 2009; VOGEL et al., 1999; YIN; SANCHETI; CADENAS, 2012). Estudos prévios demonstraram que quando a mitocôndria está energizada e as concentrações de NADPH altas, o processo de abertura do PTP é inibido; em contrapartida, quando o NADP está oxidado a abertura do PTP é favorecida (CASTILHO et al., 1995; VERCESI, 1987). Portanto, não podemos excluir a possibilidade de que em condições de disfunção mitocondrial, a depleção de NADPH causada pelo modo reverso da NNT pode potencialmente induzir a transição de permeabilidade mitocondrial, agravando a disfunção mitocondrial causada por Cis-5 e Myr. Por outro lado, uma vez que desacopladores comumente provocam oxidação do NADH, não podemos descartar que o efeito desacoplador de Cis-5 e Myr também pode contribuir para levar a enzima NTT a atuar no seu modo reverso.

Nossos resultados *in vitro* obtidos em preparações mitocondriais foram reproduzidos por métodos *in situ* em sistemas celulares, consistindo de fibras musculares esqueléticas e cardíacas, bem como de células cardíacas e hepáticas que permitem avaliar de forma mais fisiológica a funcionalidade mitocondrial em um sistema celular (KUZNETSOV et al., 2008). Observamos que o Cis-5 e o Myr comprometeram a bioenergética cardíaca nas fibras musculares, tanto quanto nos cardiomiócitos, bem como nos hepatócitos e fibras musculares esqueléticas, ao provocarem uma inibição do estado 3 e da respiração desacoplada. É importante enfatizar que os nossos resultados em células foram obtidos em condições experimentais com uma concentração de albumina baixa, similar àquela encontrada

no citosol hepático (BARAONA et al., 1977), implicando que os efeitos causados sejam atribuídos às suas formas livres ativas.

É difícil estabelecer a relevância fisiopatológica dos achados do presente trabalho porque as concentrações de Cis-5 e Myr nos tecidos dos pacientes deficientes de VLCAD ainda são desconhecidas. Entretanto, deve ser notado que os efeitos mitotóxicos foram observados com concentrações patológicas desses ácidos graxos acumulados similares àquelas encontradas no plasma dos pacientes afetados durante descompensação metabólica (5 a 500 µM) (COSTA et al., 1998; LAFORET et al., 2009). Ao contrário, concentrações fisiológicas do Cis-5 e do Myr não alteraram os parâmetros testados, ressaltando sua toxicidade somente em concentrações patológicas. Vale ainda a pena mencionar que o coração, o músculo esquelético e o fígado utilizam principalmente como fonte de energia os ácidos graxos, sendo que as enzimas do catabolismo dos LCFA estão muito expressas nesses tecidos (OEY et al., 2005; OLPIN, 2013).

O presente trabalho reforça a hipótese de que o acúmulo de Cis-5 e Myr prejudica funções mitocondriais cruciais, que pode contribuir para os fenótipos clínicos característicos dos pacientes afetados pela VLCADD. É concebível que o dano das funções mitocondriais causados pelos Cis-5 e Myr pode ser maior *in vivo* em situações de estresse metabólico, em que as concentrações desses metabólitos no meio intracelular são superiores às encontradas no plasma, já que estão acumulados dentro da célula, em pacientes afetados por essa doença. Nesse sentido, foi verificado que as concentrações das acilcarnitinas de cadeia longa acumuladas no modelo animal da VLCADD são maiores no fígado e músculo esquelético do que no plasma dos animais nocaute (SPIEKERKOETTER et al., 2005).

Finalmente, postulamos que o déficit energético na VLCADD, além de ser causado pela falta de oxidação dos LCFA em tecidos ricos em mitocôndrias (coração, fígado e músculo esquelético), é agravado pela lipotoxicidade dos principais ácidos graxos acumulados nessa doença, inibindo propriedades cruciais da mitocôndria e que esses mecanismos estão envolvidos na sua patogênese.

4. CONCLUSÕES

O Cis-5, principal marcador bioquímico usado para o diagnóstico da VLCADD, e o Myr prejudicam a bioenergética mitocondrial e a homeostase do Ca^{2+}, atuando como potentes inibidores metabólicos, desacopladores, comprometendo a OXPHOS, além de serem indutores do PTP e causar a liberação de citocromo *c* que pode levar a apoptose em músculo cardíaco e esquelético, bem como em fígado de ratos jovens (figura 7).

No que se refere à respiração celular, observamos que ambos ácidos graxos causam diminuição no estado 3, estado desacoplado e RCR, além de aumentar o estado 4, utilizando vários substratos, bem como também foram capazes de diminuir a produção de ATP em mitocôndrias de coração, fígado e músculo esquelético.

Nossos resultados *in vitro* obtidos das frações mitocondriais foram reproduzidos em sistemas celulares (fibras cardíacas e musculares esqueléticas, cardiomiócitos e hepatócitos) que mais mimetizam o meio intracelular *in vivo*.

Também verificamos que o Cis-5 e o Myr diminuem o potencial de membrana e a capacidade de retenção de cálcio em mitocôndrias de coração, fígado e músculo esquelético, assim como o conteúdo de NAD(P)H em coração e causam inchamento mitocondrial com a liberação de citocromo *c* em fígado. Alguns desses efeitos foram prevenidos pelos inibidores da abertura do PTP, indicando que esse mecanismo está envolvido nos efeitos observados.

Além disso, observamos que ocorreu uma inibição do complexo I da cadeia respiratória em mitocôndrias de coração e do complexo I-III em fígado e músculo esquelético, e também da enzima α-cetoglutarato desidrogenase em fígado, o que pode explicar em parte as inibições observadas na respiração mitocondrial.

Não houve alterações nas outras atividades enzimáticas e complexos testados, nem na fluidez de membrana.

Propomos que a mitotoxicidade causada por esses ácidos graxos que mais se acumulam na VLCADD pode estar associada com as alterações de bioenergética mitocondrial encontradas em tecidos de camundongos do modelo genético animal de VLCADD.

Presumimos que nossos achados podem estar associados com as manifestações cardíacas, hepáticas e musculares que ocorrem nos pacientes afetados pela VLCADD, especialmente durante crises metabólicas caracterizadas por um aumento significativo dos ácidos graxos acumulados nos tecidos dos pacientes.

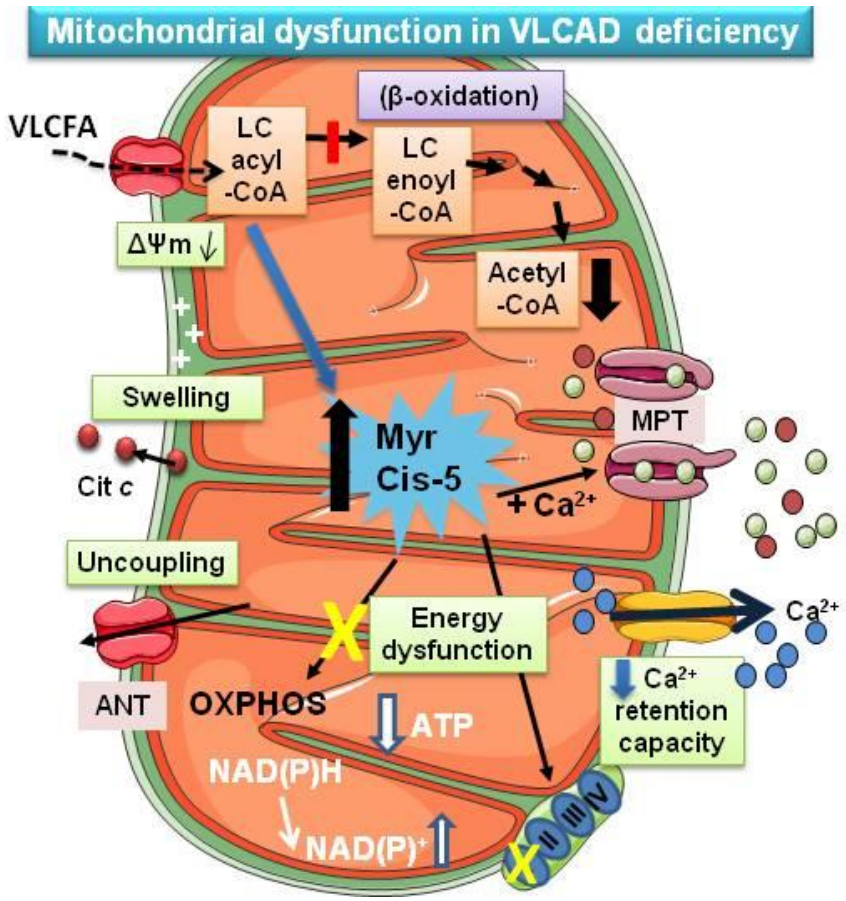

Figura 7. Disfunção mitocondrial na deficiência da VLCAD. O bloqueio da β-oxidação causado pela deficiência da VLCAD leva ao acúmulo de ácidos graxos, principalmente Myr e Cis-5. Esses metabólitos causam diminuição do ΔΨm, inchamento mitocondrial, liberação de citocromo *c,*

desacoplamento com auxílio do ANT. Causam disfunção energética com inibição da OXPHOS, atividades dos complexos I e I-III, diminuição da produção de ATP e do conteúdo de NAD(P)H, além de induzirem a abertura do PTP (MPT) e diminuir a capacidade de retenção de Ca^{2+}. Fonte: própria autoria.

5. PERSPECTIVAS

- Avaliar a bioenergética mitocondrial nos moldes do presente trabalho em vários tecidos de camundongos nocaute para a VLCAD, bem como em fibroblastos e músculo esquelético de pacientes portadores da VLCADD;
- Avaliar o papel das acilcarnitinas de cadeia longa e dos seus derivados acil-CoA, que também são encontrados em altas concentrações no sangue desses pacientes, sobre a homeostase mitocondrial;

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