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“Biochemical studies on the physiopathology of MCAD, LCHAD and MTP deficiencies in human fibroblasts and rodent tissues: evidence of disruption of redox and energy homeostasis by accumulated metabolites"

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The current Thesis was submitted to the Postgraduate Program in Biological Science - Biochemistry, of the Universidade Federal do Rio Grande do Sul, Brazil and to the Faculty of Health of the Aarhus University, Denmark in partial fulfillment of the requirements for PhD degree approval

Porto Alegre, 2014

Acknowledgements

I would like to express my sincere gratitude to my advisor Prof. Moacir Wajner for the continuous support of my PhD study and research, for the encouragement to the PhD-Sandwich and for the double degree, and especially for his patience and caring.

Besides my advisor, I would like to thank my co-supervisor Niels Gregersen for receiving me so well in his lab and for 10 months of learning in Denmark. I also thank all my labmates at MMF/ Denmark, for all support and friendship.

I would like to thank my labmates and friends from the labs 38 and 27 for all help, friendship and for all the fun we have had in the last four years.

Last but not the least, I would like to thank my family and boyfriend for all love and support.

To my family

Summary

SECTION I –

INTRODUCTION AND PURPOUSE

Abstract

The medium chain acyl-CoA dehydrogenase (MCAD) deficiency is a fatty acid oxidation disorder (FAOD) biochemically characterized by accumulation of medium-chain acylcarnitines (MCAC) in tissues and biological fluids of affected individuals. The clinical presentation includes neurological symptoms as lethargy and coma, generally followed by episodes of metabolic decompensation. Other important FAOD are the mitochondrial trifunctional protein (MTPD) and the long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHADD) deficiencies that are characterized by accumulation of long-chain fatty acids and their 3-hydroxylated (LCHFA) derivatives. Usually, affected patients present cardiac and hepatic dysfunction and may present signs of neurological impairment. So far, hypoglycemia and the toxicity of accumulating fatty acids have been related with the pathophysiology in these disorders, although the mechanisms responsible for tissue damage are poorly known. Thus, we investigated the effects of MCAC and LCHFA on important parameters of mitochondrial redox and energetic homeostasis in brain and heart of rats, as well as superoxide production and cell death in skin fibroblasts from patients affected by these diseases. First, we observed that MCAC induced lipid and protein oxidative damage, and reduced the antioxidant non-enzymatic defenses in rat brain, probably through induction of hydroxyl and peroxyl radicals. Furthermore, we evidenced increased superoxide levels and cell death in skin fibroblasts from MTP deficient patients under standard growing conditions indicating a chronic exposure to higher superoxide levels and a vulnerability to cell death. In addition, cells from MCADD and MTPD patients cultured under metabolic stress conditions presented increased levels of superoxide, although cell death was not increased. Finally, cell death was more pronounced in fibroblasts from MTP compared to MCAD deficient patients, and presented a strong correlation with superoxide production, suggesting that these events are probably associated. On the other hand, we observed that LCHFA provoked an impairment of heart mitochondrial energetic homeostasis, by behaving as uncouplers of oxidative phosphorylation, evidenced by increase of state 4 respiration and decrease of the respiratory control ratio, by reducing the membrane potential, the NAD(P)H content and H_2O_2 production. 3-Hydroxytetradecanoic acid (3 HTA) also induced mitochondrial swelling probably as a consequence of the mitochondrial permeability transition pore (mPTP) opening once cyclosporin A (CsA), a classical inhibitor, prevented this effect in heart mitochondria loaded with Ca^{2+}. LCHFA also dissipated membrane potential, diminished NAD(P)H content and decreased ATP content in the presence or absence of Ca^{2+} in cerebral cortex mitochondrial preparations. Moreover, 3 HTA induced mitochondrial swelling and cytochrome *c* release in the presence of Ca^{2+}, which activates apoptotic cascades. 3 HTA also affected Ca^{2+} homeostasis evidenced by a diminished mitochondrial Ca^{2+} retention capacity and induced hydrogen peroxide production in the presence of Ca^{2+}. These alterations where prevented by ruthenium red (RR), a blocker of mitochondrial Ca^{2+} uptake, and by CsA plus ADP, inhibitors of the mPTP, implying its involvement in these effects. In contrast, LCHFA did not cause oxidative damage nor altered the antioxidant defenses in heart of young rats. Taken together, we demonstrated that MCAC and LCHFA found accumulated in patients affected by MCAD and MTP/LCHAD deficiencies, respectively, are potentially toxic to essential mitochondrial functions in rat brain and heart. We also found that fibroblasts from MTP deficient patients cultured under basal growing conditions are under oxidative stress that is accentuated when cultured metabolic stress conditions are used. It is presumed that these pathomechanisms may be responsible, at least in part, for the tissue damage characteristic of these patients.

Resumo

A deficiência da desidrogenase de acilas-CoA de cadeia média (MCAD) é um defeito da oxidação de ácidos graxos (DOAG) bioquimicamente caracterizada pelo acúmulo de acilcarnitinas de cadeia média (MCAC) em tecidos e líquidos biológicos de indivíduos afetados. Clinicamente os pacientes apresentam sintomas neurológicos como letargia e coma, geralmente desencadeados por episódios de descompensação metabólica. Outros distúrbios importantes entre os DOAG são as deficiências da proteína trifuncional mitocondrial (MTPD) e da desidrogenase de 3-hidroxiacilas-CoA de cadeia longa (LCHADD) que são caracterizadas pelo acúmulo de ácidos graxos de cadeia longa e seus derivados 3-hidroxilados (LCHFA). Geralmente, os pacientes afetados apresentam disfunção cardíaca e hepática podendo também apresentar sinais de comprometimento neurológico. Até o momento, a hipoglicemia e a toxicidade dos ácidos graxos acumulados têm sido relacionados com a fisiopatologia dessas doenças, embora os mecanismos responsáveis pelo dano tecidual não são bem conhecidos. Assim, foram investigados os efeitos dos MCAC e LCHFA sobre importantes parâmetros da função redox e homeostase energética mitocondrial em cérebro e coração de ratos, bem como a produção de superóxido e morte celular em culturas de fibroblastos da pele de pacientes afetados por essas doenças. Primeiramente, observamos que as MCAC induzem dano oxidativo lipídico e proteico, além de reduzir as defesas antioxidantes não enzimáticas em cérebro de ratos, provavelmente através da indução de radicais hidroxil e peroxil. Além disso, evidenciamos aumento dos níveis de superóxido e de morte celular em fibroblastos de pacientes afetados pela MTPD em condições padrão de cultivo indicando uma exposição crônica a níveis mais elevados de superóxido e uma vulnerabilidade à morte celular. Fibroblastos de pacientes afetados pelas MTPD e MCADD também apresentaram aumento dos níveis de superóxido quando cultivadas em condições de estresse metabólico, contudo não houve aumento de morte celular. Finalmente, a morte celular foi mais pronunciada em fibroblastos afetados pela MTPD em comparação com pacientes afetados pela MCADD, além de apresentar uma forte correlação com a produção de superóxido, sugerindo que esses eventos são provavelmente associados. Por outro lado, observamos que LCHFA prejudicam a homeostase energética em mitocôndrias de coração devido a um efeito desacoplador da fosforilação oxidativa evidenciado pelo aumento do estado 4 da respiração e diminuição da razão de controle respiratório, pela redução do potencial de membrana, do conteúdo de NAD(P)H e da produção de H_2O_2. O ácido 3- hidroxitetradecanóico (3 HTA) também induziu inchaço mitocondrial provavelmente como consequência da abertura do poro de transição de permeabilidade mitocondrial (mPTP) uma vez que a ciclosporina A (CsA), um inibidor clássico da abertura do poro, impediu esse efeito em mitocôndrias cardíacas carregadas com Ca^{2+}. LCHFA também dissipadaram o potencial de membrana, diminuíram o coneúdo de NAD(P)H e de ATP na presença ou ausência de Ca^{2+} em mitocondriais de córtex cerebral. Além disso, na presença de Ca^{2+}, 3 HTA induziu inchamento mitocondrial e liberação de citocromo c, que ativa rotas apoptóticas. 3 HTA também afetou a homeostase de Ca^{2+} evidenciado por uma reduzida capacidade de retenção de Ca^{2+} pela mitocôndria e induziu a produção de H_2O_2 na presença de Ca^{2+}. Essas alterações foram prevenidas pelo vermelho de rutênio (RR), um bloqueador da captação mitocondrial de Ca^{2+}, e por CsA mais ADP, inibidores da abertura do mPTP, o que implica o seu envolvimento nesses efeitos. Em contraste, LCHFA não causaram dano oxidativo nem alteraram as defesas antioxidantes em coração de ratos jovens. Em conjunto, nós demonstramos que MCAC e LCHFA que se acumulam em pacientes afetados pelas deficiências da MCAD e MTP/LCHAD, respectivamente, são potencialmente tóxicos para as funções mitocondriais essenciais em cérebro e coração de ratos. Além disso, demonstramos que fibroblastos de

pacientes com MTPD cultivados em condições padrão estão sob estresse oxidativo, que é acentuado quando os fibroblastos são submetidos a condições de estresse metabólico. Presume-se que esses mecanismos fisiopatológicos podem ser responsáveis, pelo menos em parte, pelo dano tecidual característico desses pacientes.

Abbreviation list

ACADs - acyl-CoA dehydrogenases

ANT - adenine nucleotide translocator

Alm - alamethicin A

AA - antimycin A

ATC - atractyloside

CCCP - carbonyl cyanide 3-chlorophenyl hydrazine

CT - carnitine-acylcarnitine translocase

CPT 1 - carnitine palmitoyl transferase 1

CPT 2 - carnitine palmitoyl transferase 2

cDC - *cis*-4-decenoylcarnitine

CoQ - coenzyme Q

CsA - cyclosporin A

DA - decanoic acid

DC - decanoylcarnitine

ETF - electron transfer flavoprotein

ETS - electron transfer system

EGTA - ethylene glycol-bis (2-aminoethylether-)-N-N-N’-N’-tetraacetic acid

FA - fatty acids

FAO - fatty acid oxidation

FAU - fluorescence arbitrary units

HC - hexanoic acid

HG - hexanoylcarnitine

3 HDCA – 3-hydroxydecanoic acid

3 HDA - 3-hydroxydodecanoic acid

HEPES – N-[2-hydroxyethyl]piperazine-N’-[2-ethane-sulfonic acid]

3 HTA - 3-hydroxytetradecanoic acid

3 HPA - 3-hydroxypalmitic acid

IEM - inborn errors of metabolism

LCHAD - long-chain acyl-CoA dehydrogenase

LCEH - long-chain enoyl-CoA hydratase

LCFA - long-chain fatty acids

LCHFA – long-chain 3-hydroxy fatty acids

LCKT - long-chain 3-ketoacyl-CoA thiolase

MCAC - medium-chain acylcarnitines

MCAD - medium-chain acyl-CoA dehydrogenase

MCT - medium-chain triglyceride

MEL - melatonin

$\Delta\Psi$ - membrane potential

mPTP - mitochondrial permeability transition pore

MTP - mitochondrial trifunctional protein

$_{L}$-NAME - N^{ω}-nitro-L-arginine methyl ester

OA - octanoic acid

OC - octanoylcarnitine

GSH - reduced glutathione

RR - ruthenium red

TRO – trolox

1. Introduction

1.1. Inborn errors of metabolism

The inherited metabolic disorders, known as inborn errors of metabolism (IEM), were first described in 1908 by Archibald Garrod, whose studies resulted in the description of the alkaptonuria, an IEM of the phenylalanine and tyrosine metabolism. So far, about 500 IEM have been biochemically and genetically characterized, affecting proteins related to process of biosynthesis, degradation, storage and transport (Scriver, 2001). The clinical phenotype of this group of metabolic diseases is therefore heterogeneous.

An early diagnosis through neonatal screening allows the introduction of treatment during the pre-symptomatic period, leading to the clinical improvement of patients even before the manifestations of the symptoms. The treatment may include a dietary restriction of some substrates, replacement of a lacking metabolic product, supplementation of a cofactor, removal of toxic metabolites, enzyme replacement therapy and, in few cases, organ transplantation (*e.g.* liver) (Scriver, 2001).

At present, the pathophysiology involved in IEM is under intense investigation since the elucidation of the underlying pathomechanisms will potentially contribute to the therapy. Different tools have been used to identify the mechanisms responsible for the development of symptoms and include animal models for some of these disorders.

1.2. Mitochondrial fatty acid oxidation

Fatty acid oxidation (FAO) is the main source of energy for the skeletal and cardiac muscle, while the liver oxidizes fatty acids (FA) especially under the conditions of catabolism as prolonged fasting and illness. Moreover, FAs also play a significant role in cell signaling, influence enzymatic activities, gene expression and ion homeostasis (Graber et al., 1994).

The oxidation of FA with carbon chain length 20 or fewer is a mitochondrial process and consists of multiple repetitive steps of four enzymatic reactions resulting in the sequential removal of two-carbon as acetyl-CoA units (Roe and Ding, 2001). First, plasma long-chain fatty acids (LCFA) are transported actively across the plasmatic membrane, then activated to acyl-CoA esters and transesterified to acylcarnitines to be transferred across the mitochondrial membrane by carnitine palmitoyl transferases 1 (CPT 1) and 2 (CPT 2) in conjunction with the carnitine-acylcarnitine translocase (CT). Inside the mitochondria, they are re-esterified to acyl-CoA esters and follow to the β-oxidation spiral. The four reactions of the β-oxidation cycle are catalyzed by acyl-CoA dehydrogenases (ACADs), enoyl-CoA hydratases, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl- CoA thiolase (Figure 1). Electrons resulting from these processes are transferred to the electron transfer flavoprotein (ETF). Within the spiral, each step is catalyzed by enzymes with overlapping chain-length specificities. Taken together, the β-oxidation process includes at least 25 enzymes and transport proteins.

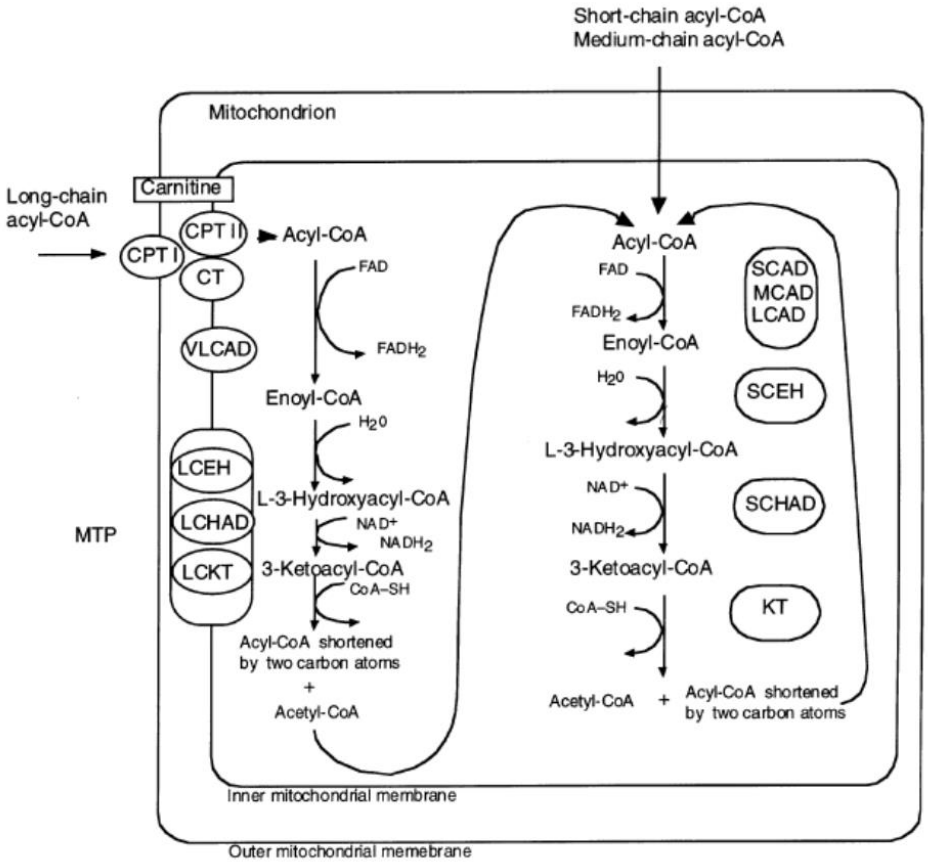

Figure 1. Scheme of mitochondrial fatty acid oxidation. Carnitine palmitoyl transferases 1 (CPT 1) and 2 (CPT 2); carnitine-acylcarnitine translocase (CT); very long-chain acyl-CoA dehydrogenase (VLCAD); mitochondrial trifunctional protein (MTP); long-chain enoyl-CoA hidratase (LCEH); long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD); long-chain 3-ketoacyl-CoA thiolase (LCKT); long-chain acyl-CoA dehydrogenase (LCAD); medium-chain acyl-CoA dehydrogenase (MCAD); short-chain acyl-CoA dehydrogenase (SCAD); short-chain enoyl-CoA hidratase (SCEH); short-chain hydroxyacyl-CoA dehydrogenase (SCHAD); ketothiolase (KT). Adapted from Tyni and Pihko (1999).

It has been described that the proteins responsible for mitochondrial FAO are physically and functionally associated with each other and with the electron transfer system (ETS) supercomplexes (Wang et al., 2010) (Figure 2). This is important since the association of FAO and ETS in the inner mitochondrial membrane optimize the efficiency of energy metabolism (Rinaldo et al., 2002; Wang et

al., 2010). Thus, FAO provides reduced equivalents directly to the ETS that are transferred via ETF to ETF dehydrogenase, resulting in reduction of coenzyme Q (CoQ) or NAD through the 3-hydroxyacyl-CoA dehydrogenase to form NADH, the substrate for complex I (Sumegi et al., 1991).

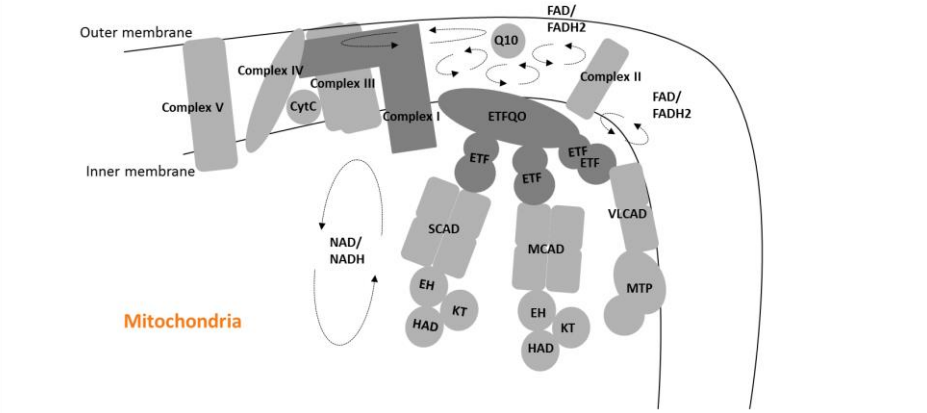

Figure 2. Scheme of physical association of mitochondrial fatty acid oxidation and oxidative phosphorylation complexes. Mitochondrial trifunctional protein (MTP); very long-chain acyl-CoA dehydrogenase (VLCAD); 3-hydroxyacyl-CoA dehydrogenase (HAD); 3-ketoacyl-CoA thiolase (KT); enoyl-CoA hidratase (EH); medium-chain acyl-CoA dehydrogenase (MCAD); short-chain acyl-CoA dehydrogenase (SCAD); electron transfer flavoprotein (ETF); electron transfer flavoprotein - ubiquinone oxidoreductase (ETFQO). Adapted from Gregersen and Olsen, 2010.

Once mobilized from adipose tissue, stored LCFA are transported in the circulation primarily bound to albumin. During periods of fasting, fatty acids become the predominant fuel for energy production in liver, cardiac and skeletal muscles. The brain does not directly utilize fatty acids for oxidative metabolism, but readily oxidizes ketone bodies produced by β-oxidation of fatty acids in the liver (Nelson and Cox, 2008).

1.3. Mitochondrial fatty acid oxidation disorders

FAO disorders are an important group of inherited metabolic diseases whose affected patients have heterogeneous clinical presentations and high morbidity and mortality. Clinical presentation in these patients usually occurs within the first years of life, especially after catabolic events with fever, vaccination, fasting and prolonged exercise. The most common manifestations during crisis are vomiting, lethargy, coma and in some cases, sudden death (Rector et al., 2008). Regarding to the genotype/phenotype correlation, it has been postulated that patients affected by loss-of-function mutations (null mutations) present a severe phenotype than those affected by missense mutations, in which some enzymatic activity is preserved. However, the second group can also present the severe phenotype making a genotype-phenotype correlation difficult.

1.3.1 Medium-chain acyl-CoA dehydrogenase deficiency

MCAD deficiency is recognized as the most common inherited FAO disorders with an estimated frequency of one in 14,600 (Rhead, 2006), mainly affecting Caucasians of northern European origin (Rhead, 2006; Wilcken et al., 1994).

MCAD deficiency is a result of a genetic recessive mutation in the *ACADM* gene, being the mutation c.985A>G the most frequent in symptomatic patients (Gregersen et al., 2004; Schatz and Ensenauer, 2010). However, after the implementation of the newborn screening for FAO diseases, other genotypes for MCAD deficiency were found with high frequency as the missense mutation c.199C>T that is associated to low risk of disease manifestation (Andresen et al., 2012). Since the development of symptomatology has been related with the residual enzyme activity, patients homozygous for some of the mutations may present symptoms, whereas, when heterozygotes, remain asymptomatic.

Children affected by MCAD deficiency often present vomiting and lethargy following energetic decompensation episodes triggered by fasting, infection or other metabolic stressors (Matsubara et al., 1992). The clinical phenotype varies from a mild to severe symptomatology including Reye's syndrome, muscle weakness, hepatic steatosis, failure to thrive, coma and sudden death. The neurological findings include cerebral edema and abnormalities in the frontal lobus, as well as global developmental disability, speech and language delay, behavioral problems, attention deficit, chronic seizure disorder and cerebral palsy (Roe and Ding, 2001).

Most children present with acute illness between 3 and 15 months of age. When undetected, approximately 20–25% of infants will die or suffer permanent neurologic impairment as a consequence of the first acute metabolic decompensation (Iafolla et al., 1994; Pollitt and Leonard, 1998; Wilcken et al., 2007; Wilcken et al., 1994). Thus, the goal of an early treatment is to prevent the development of symptoms (Kompare and Rizzo, 2008).

Biochemically, patients present hypoketotic hypoglycemia and characteristic accumulation of medium-chain acylcarnitines (MCAC) and acylglycines in plasma and dicarboxylic acids in urine, which are used as markers for diagnosis.

Table 1. Blood concentrations (μmol/L) of intermediates of fatty acid oxidation in plasma of individual patients affected by MCADD and controls under different metabolic conditions

Metabolite	**Normal individuals**	**Individuals affected by MCADD**
Blood concentration (μM)		
Octanoylcarnitine[e]	<0,17	0,6-36,7

Hexanoylcarnitine[c]	<0,21	0,13-4
Decanoylcarnitine[c]	<0,30	0,11-2,6
Decenoylcarnitine[c]	<0,04	0,13-1,7
Urine (μg/mg creatinine[1]; μmol/mmol creatinine[2])		
Hexanoylglycine[1,d]	0,21-1,9	3,1-653
Fenilpropionylglycine[1,d]	0-1,1	1,2-180
Suberylglycine[1,c]	0-95	13-4553
Adipic acid[,a]	-----	1,5-150
Suberic acid[2,a]	-----	0-214

Adaptated from [a](Downing et al., 1989), [b](Onkenhout et al., 1995), [c](Chace et al., 1997), [d](Rinaldo et al., 1998), [e](Rhead, 2006).

1.3.1.1 Diagnosis

The characteristic profile of acylcarnitines in MCAD deficiency in blood is carried out by tandem mass spectrometry (MS/MS) and includes high concentrations of hexanoylcarnitine, octanoylcarnitine and cis-4-decenoylcarnitine (Pandor et al., 2004). Urinary analyses reveal the characteristic acylglycine elevations (hexanoyl- and suberyl-glycine). Unlike the urinary organic acids profile, the plasma acylcarnitine profile is diagnostic in both sick and asymptomatic children with MCAD deficiency allowing the detection of patients prior to the onset of symptoms. The diagnosis confirmation is made by enzymatic and mutational analyses in lymphocytes or fibroblasts from patients.

1.3.1.2 Management and treatment

The management of MCAD-deficient patients includes frequent feeding, adequate caloric intake, low-fat diet (20% of total caloric intake), avoidance of fasting and intravenous glucose therapy during acute episodes (Roe and Ding, 2001). L-carnitine is also supplemented and constitutes in an useful conjugation pathway for the removal of intermediates that accumulate in the tissues of patients under catabolic stress conditions.

1.3.1.3 Physiopathology in MCAD deficiency

MCAD deficiency impairs the use of fatty acids as fuels and the production of ketone bodies in the liver. Thus, hypoglycemia develops most likely as a result of high glucose utilization, once ketones and fatty acids are unavailable to substitute glucose as metabolic fuels in some organs.

During fasting, fatty acids are recruited from the adipose tissue increasing their circulating levels and leading to their accumulation in non-adipose tissues and becoming cytotoxic, a condition named lipotoxicity. Additionally, the higher production of acyl-CoA intermediates in mitochondria results in a decreased CoA availability for other mitochondrial reactions.

Thus, the acyl-CoA:CoA ratio increases inhibiting the enzymes pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. Thus, the conversion of pyruvate to acetyl-CoA as well as the citrate synthesis and the flux from α-ketoglutarate to succinyl-CoA are lower impairing the functioning of the tricarboxylic acid cycle (TCA). Additionally, as citrate serves as a way of acetyl-CoA transport to the cytosol, decreased availability of citrate influences the regulation the gluconeogenesis and FAO. Citrate, when in sufficient quantities in the mitochondria, is transported to the cytosol and converted by citrate lyase into acetyl-CoA and oxaloacetate. Oxaloacetate is then converted to malate or

phosphoenolpyruvate ending in glucose production through the gluconeogenesis process. The acetyl-CoA generated from citrate in the cytoplasm is substrate for the acetyl-CoA carboxylase to the synthesis of malonyl-CoA, the primary regulator of CPT 1. Reduced malonyl-CoA levels allow unregulated entry of fatty acids into mitochondria and, in MCAD deficiency, increased production of medium chain acyl-CoA intermediates. Inadequate mitochondrial acetyl-CoA also affects pyruvate carboxylase activity because acetyl-CoA serves as an activator of this biotin-dependent enzyme, which converts pyruvate to oxaloacetate and is critical for gluconeogenesis. The impairment in gluconeogenesis may also account for the hypoglycemia seem in patients affected by MCAD deficiency (Scriver, 2001).

On the other hand, accumulating evidence has revealed that the accumulating metabolites in MCAD deficient patients present toxic properties. Thus, octanoate infused in rabbits provoked hepatic encephalopathy and fat liver deposit, many of the pathological findings of Reye's syndrome (Trauner, 1982; Trauner and Huttenlocher, 1978). It is presumed that the encephalopathy and cerebral edema observed in MCAD deficiency could result from similar mechanisms and the inhibition of the Na^+,K^+-ATPase activity caused by octanoate infusion in rabbits may be considered as a possible mechanism for the encephalopathy. Supporting this hypothesis a study has shown that acyl-compounds with three or more carbons have significant encephalopathic properties and the longer chain FA were demonstrated to cause the most significant effects, including coma (Dahl et al., 1956).

Although hypoglycemia is a common finding in MCAD deficiency, acute episodes with coma may be determined by other mechanisms than by low blood glucose levels, and this is in accordance with the observations that encephalopathic crises may occur despite correction of hypoglycemia. Therefore, other causes than hypoglycemia may represent contributor factors to the pathophysiology in inherited FAO disorders affecting mitochondrial functions (Gregersen and Olsen, 2010; Schmidt et al.,

2010; Tyni et al., 1996; Zolkipli et al., 2011). Previews studies have also shown that octanoic acid (OA) increases oxygen consumption and CO_2 production without corresponding increase in ATP production in rat liver (Berry et al., 1983; Scholz et al., 1984). Moreover, OA inhibits the control volume in astrocyte and Na^+,K^+-ATPase activity in cultured glial cells (Olson et al., 1989) what could be related with the cerebral edema found in patients. Furthermore, *in vitro* studies demonstrated that OA, decanoic (DA) and c*is*-4-decenoic acids inhibit important parameters of energy metabolism in cerebral cortex of young rats including the activities of the respiratory chain, creatine kinase and Na^+,K^+-ATPase, as well as compromise the TCA function (de Assis et al., 2006; de Assis et al., 2003; Reis de Assis et al., 2004). Moreover, it was verified that OA and DA decreased the activity of the respiratory chain in rat liver and skeletal muscle (Scaini et al., 2012). It was also shown that MCFA induced lipid peroxidation, enhanced protein oxidative damage and decreased the non-enzymatic antioxidant defenses in rat cerebral cortex, skeletal muscle and liver (Scaini et al., 2012; Schuck et al., 2007; Schuck et al., 2009). Taken together, these findings indicate disturbances of mitochondrial energy and redox homeostasis caused by the major metabolites accumulating in MCADD.

Besides energy deficiency and the toxicity of accumulating fatty acids, the pathophysiology in MCAD deficiency may also be related with the presence of misfolded mutant proteins that can result from missense mutations (Gregersen, 2006; Gregersen and Bross, 2010; Gregersen et al., 2006; Schmidt et al., 2010). Since it has been shown that mutant medium-chain and short-chain acyl-CoA dehydrogenases as well as ETF and ETF dehydrogenase may accumulate during cellular stress, there is speculation about how such proteins may disturb mitochondrial functioning (Figure 2) (Gregersen and Olsen, 2010). So far, the consequences for electrons and hydrogen transfer, when one of the ACADs is absent due to null mutations, or misfolded and aggregated inside the mitochondria as a consequence of missense mutations, is not clear. In the first case, electrons and hydrogen could flow through the other

ACADs with overlapping substrate specificity, even though it might result in substrate accumulation and lack of substrate to energy production. Whereas one ACAD was inactive or misfolded, it could impair the activity of others ACADs and provoke an even higher accumulation of metabolites. In this way, such mechanism could take part in patients affected by the common missense mutation c.985A > G that is more severe (presence of symptoms in patients) than null mutations (Gregersen et al., 2008; Gregersen and Olsen, 2010).

1.3.2 Mitochondrial trifunctional protein deficiency

Mitochondrial trifunctional protein (MTP) complex is formed by four α and four β subunits encoded by *HADHA* and *HADHB* genes. MTP complex participates in the last three steps in long-chain FAO process and comprehends three enzyme activities. The α subunit is encoded by *HADHA* gene and present the activities of long-chain enoyl-CoA hydratase (LCEH) and long-chain acyl-CoA dehydrogenase (LCHAD), whereas *HADHB* gene encodes the β subunit with 3-ketoacyl-CoA thiolase (LCKT) activity. MTP deficiency is caused by mutations in either one or both genes that encode MTP complex, affecting all three enzyme activities. Moreover, isolated mutation in *HADHA* gene can cause defects in LCHAD activity with normal or slightly reduced activity of the other two enzymes (Ushikubo et al., 1996) .

So far, in MTP deficiency there is no clear prevalent mutation. The described patients are affected by different mutations in one and/or the two subunits of the MTP, destabilizing the whole complex and resulting in general MTP deficiency. As regards to the clinical presentation, patients may have the lethal cardiomyopathic form, an infant-onset hepatic form or a late-onset neuromyopathic form. The lethal phenotype is characterized by severe dilated cardiomyopathy, lactic acidosis, Reye-

like syndrome, hypoketotic hypoglycemia and neonatal death. The hepatic phenotype occurs within the first months of life, also presenting with episodes of hypoketotic hypoglycemia, lactic acidemia and lethargy. The late-onset neuromyopathic form is characterized by progressive peripheral neuropathy and episodic of myoglobinuria (Spiekerkoetter et al., 2004a; Spiekerkoetter et al., 2004b).

Furthermore, patients may become anorexic, lethargic, severely hypotonic, areflexic and unresponsive to pain. Biochemically, patients present accumulation of long-chain fatty acids and their hydroxyl intermediates in tissues and biological fluids (Costa et al., 1998).

1.3.2.1 Diagnosis

The MTP deficiency is difficult to recognize and identify by the usual methods for diagnosis of FAO disorders. Urine organic acid analysis may or not show dicarboxylic and 3-hydroxydicarboxylic aciduria (Scriver, 2001). Unlike isolated LCHAD deficiency, there is no common mutant allele and the α subunit 1528G > C mutation is not observed in complete MTP deficiency. The MTP deficiency seems to be the only FAO disorder in which blood lactate is constantly elevated even when patients are asymptomatic, suggesting mitochondrial bioenergetics impairment. At present, there is no simple direct tool to rapidly confirm this defect. Thus, an enzymatic assay is necessary for diagnosis (Spiekerkoetter et al., 2004b).

1.3.2.2 Treatment

The treatment of MTP-deficient patients consists in reduced LCFA diet, carbohydrate-enriched feedings or supplementation with uncooked cornstarch. In addition, medium-chain triglycerides (MCT) supplementation seems to be helpful to the improvement of the patients´ conditions.

1.3.2.3 Physiopathology in MTP deficiency

Until the present, information regarding the physiopathology in MTP deficiency is relatively limited. However, we cannot rule out that energy deficiency associated with other contributor factors are the responsible for the cardiomyopathy found in MTP-deficient patients. In this regard, Brown and O'Rourke (2010) observed arrhythmogenic effects of long-chain acylcarnitines. Moreover, skeletal myopathy can be prevented with MCT supplementation prior to exercise in MTP- and LCHAD-deficient patients (Gillingham et al., 2006).

In this context, experimental evidences point to deleterious effects of long-chain fatty acids and their derivatives on energy metabolism. It was shown that inhibition of ATP synthesis by palmitoyl-CoA and its 3-hydroxy and 3-keto derivatives in human fibroblasts (Ventura et al., 1995; Ventura et al., 1996). Moreover, long chain acyl-CoA esters inhibited the oxidative phosphorylation, the mitochondrial ATP/ADP translocator and the dicarboxylate carrier, as well as respiratory chain enzyme activities in isolated rat liver mitochondria (Ventura et al., 2005; Ventura et al., 2007). Furthermore, our group has recently reported that LCHFA acts as uncouplers of oxidative phosphorylation, impairing mitochondrial respiration and reducing the matrix NAD(P)H pool in rat forebrain (Tonin et al., 2013). Moreover, other *in vitro* studies elicited lipid and protein oxidative damage provoked by LCHFA, as well as decrease the non-enzymatic antioxidant defenses in rat brain (Tonin et al., 2010). Taken together, these findings could explain the lactic acidemia found in patients, once the accumulating long-chain 3-hydroxyacyl-CoA and their intermediates probably disrupt mitochondrial bioenergetics (den Boer et al., 2002; Ventura et al., 1998).

However, other complications found in these patients such as peripheral neuropathy and retinopathy is not reversible with treatment, suggesting the involvement of other pathomechanisms besides energetic deficiency.

1.3.3 Long-chain 3-hydroxyacyl-Coa dehydrogenase

Isolated LCHAD deficiency is recognized as one of the most severe FAO disorders (Wanders et al., 1989). About half of the patients die, either from the first episode or with progressive disease ending in cardiorespiratory failure. Although there is generally a high mortality and morbidity, but the disorder can also be consistent with long-term survival in patients identified and treated from an early age (den Boer et al., 2002; Olpin et al., 2005; Tyni et al., 1997).

As described in section 1.3.1.3, patients affected by LCHAD deficiency may also develop symptomatology usually preceded by a catabolic episode. The major manifestations include hypoglycemia, hypertrophic cardiomyopathy, fulminant hepatic disease, hypotonia and rhabdomyolysis. Long-term symptomatology include neuropathy and pigmentary retinopathy (Spiekerkoetter et al., 2004b). Neurologically, early psychomotor development can be normal but patients tend to lose skills with episodes of metabolic illness and present the characteristic late onset with speech and developmental delay that reflects cerebral dysfunction (Waisbren et al., 2013). The age of onset of first symptoms ranges from 1 day to 39 months.

LCHAD deficiency is associated with the common missense mutation 1528G > C that is located at the catalytic site of the LCHAD domain. It results in intact mutant protein with significantly reduced LCHAD activity whereas the activities of LCKT and LCEH, remains at >60% of normal. This mutation is present in homozygous form in about 70 % of affected individuals.

Biochemically, patients excrete large quantities of 3-hydroxy dicarboxylic acids as well as medium-chain dicarboxylic acids in urine. Moreover, the acylcarnitine derivatives of the 3-hydroxydocecanoate, 3-hydroxytetradecanoate and 3-hydroxypalmitate monocarboxylic acids have been observed routinely by tandem-MS in blood or plasma from patients with LCHAD deficiency.

1.3.3.1 Diagnosis

Acylcarnitine analysis may present the characteristic 3-hydroxylated fatty acids 3-hydroxydodecanoic (3 HDA), 3-hydroxytetradecanoic (3 HTA) and 3-hydroxypalmitic (3 HPA) acids. Urinary analysis also shows large quantities of 3-hydroxydicarboxylic acids of 6 to 14 carbons, as well as medium-chain dicarboxylic acids that are often the clue to the diagnosis of LCHAD deficiency. Mutational analysis for the common mutation 1528G > C can confirm the diagnosis.

LCHAD activity in fibroblasts is usually assayed using 3-ketoacyl-CoA compounds such as 3-ketopalmitoyl-CoA as substrate. Because of the overlapping substrate specificities of LCHAD and SCHAD, most patients with LCHAD deficiency have demonstrated 15 to 35 % of activity compared to controls.

1.3.3.2 Treatment

Treatment of patients with LCHAD deficiency is similar to treatment of patients affected by the MTP deficiency and is described in section 1.3.2.2.

1.3.3.3 Physiopathology in LCHAD deficiency

As discussed in section 1.3.1.3 (Physiopathology in MCAD deficiency) energy deficiency caused by FAO impairment and accumulation of fatty acids have been related with the severity of symptoms found patients affected by FAO disorders. Additionally, in section 1.3.2.3 (Physiopathology in MTP deficiency) we described the toxic effects of LCHFA that accumulate in MTP and LCHAD deficiencies.

Pathological findings include fat accumulation in liver, skeletal muscle and heart of affected patients (Roe and Ding, 2001). Liver electron microscopy also showed mitochondrial abnormalities similar to those found in MCAD- and VLCAD- deficient patients, including condensed mitochondrial matrixes. In a few cases, the liver was reported to be fibrotic or frankly cirrhotic, which is not a typical feature of FAO disorders. Muscle biopsies from LCHAD deficient patients also showed irregular mitochondrial morphology with swollen appearance containing fat infiltration (Tyni et al., 1996). Skeletal muscle present also a predominance of type II fibers (slow oxidative) and necrosis associated with elevated creatine phosphokinase levels consistent with a rhabdomyolytic process. Dilated cardiomyopathy and hepatic steatosis are also prominent findings.

1.4. Mitochondria and oxidative phosphorylation

Mitochondria are membrane bound organelles present in almost all eukaryotic cells. Besides being responsible for cellular energy metabolism, they also contribute in many process including calcium metabolism, cell growth and differentiation as well as cell death. Furthermore, mitochondria are the main site of superoxide and other reactive species generation in the cell.

Oxidative phosphorylation is the process in which molecular O_2 is reduced to H_2O with electrons from NADH and $FADH_2$ that flow through the electron transport system (ETS). The ETS is

comprised of several enzymes and the fat-soluble CoQ or ubiquinone (Di Donato, 2000). Complex I, known as NADH dehydrogenase and NADH ubiquinone oxidoreductase, transfers electrons from NADH to ubiquinone. The complex II (succinate dehydrogenase) reduces ubiquinone with electrons from $FADH_2$ oxidation formed from succinate to fumarate conversion in the citric acid cycle. The complex III or cytochrome bc1 ubiquinone - cytochrome *c* oxidoreductase - catalyzes the reduction of cytochrome *c* from reduced ubiquinone. At the end of the electron transport chain, complex IV (cytochrome *c* oxidase) catalyzes the transfer of electrons from reduced molecules of cytochrome *c* to O_2, forming H_2O. Thus, this electron transfer is driven by an increasing redox potential between the reduced equivalents (NADH and $FADH_2$), the enzymatic complexes of the electron transport chain and O_2, which is the final electron acceptor. It takes four molecules of cytochrome *c* to completely reduce one molecule of O_2. All these complexes have specific prosthetic groups to play the role of electron donors and acceptors (Abeles et al., 1992).

The energy generated from the electrons flow through the ETS is accompanied by pumping of protons across the inner mitochondrial membrane (from the mitochondrial matrix to the intermembrane space) through complexes I, III and IV. This process generates the membrane potential in the form of a pH gradient and an electric potential across the membrane that is used by the ATP synthase, which allow the protons to flow back to the matrix, driving the synthesis of ATP from ADP and inorganic phosphate (Pi) (Nelson and Cox, 2008). Therefore, the electron flow and protons pumping are coupled to the ATP synthesis process, *i.e.* when the $\Delta\Psi$ is dissipated by the flow in favor of the proton electrochemical gradient, the released energy is used by ATP synthase for the synthesis of ATP (Nelson and Cox, 2008).

The outer mitochondrial membrane is readily permeable to small molecules and ions, whereas the inner membrane is impermeable to most molecules and ions, including protons and molecules of

NADH or $FADH_2$. Thus, this molecules require transfer systems to pass from the cytosol to the mitochondrial matrix (Nelson and Cox, 2008). In this context, the mitochondrial oxidation of the cytosolic NADH is allowed by systems called shuttles that transfer electrons from NADH from the cytosol to the matrix via molecules capable of being transported across the inner mitochondrial membrane. For this purpose, there are two shuttles called malate/aspartate shuttle and glycerol- 3-phosphate shuttle that are illustrated in Figure 3.

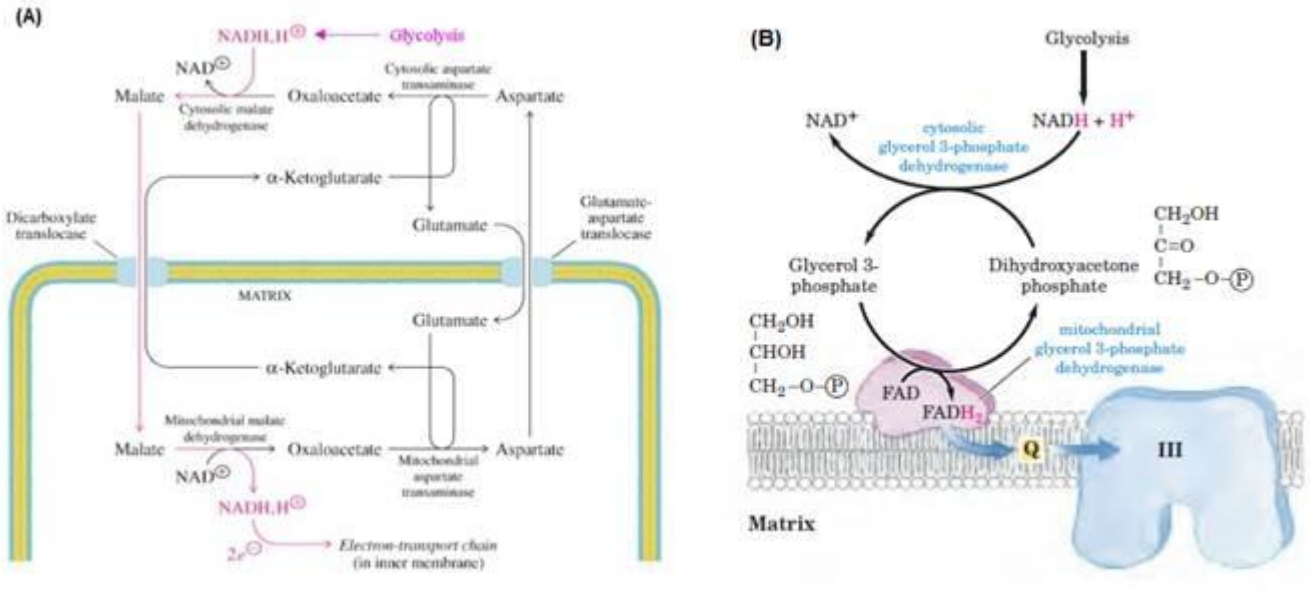

Figure 3. Malate/aspartate shuttle (A) and glycerol -3- P shuttle (B) (Nelson and Cox, 2005).

Thus, oxygen supports mitochondrial respiration also called oxidative phosphorylation and is used to form the mitochondrial transmembrane gradient potential that drives much of the synthesis of ATP, the transfer of calcium and other ion exchangers and import of proteins.

Mitochondrial respiration can be measured by O_2 consumption. Various steps can be investigated, such as the transport of substrates across the mitochondrial membrane, the activity of dehydrogenases, the activity of respiratory chain complexes, the transport of adenine nucleotides through the mitochondrial membrane, the activity of ATP synthase and mitochondrial membrane permeability to H^+ (Nicholls and Ferguson, 2002). Experimentally, mitochondrial respiration can be divided into five respiratory stages (Figure 4). However, only the parameters named states 3 and 4 are commonly used. The state 3 is characterized by high oxygen consumption in a medium containing energetic substrates and ADP, stimulating O_2 consumption and ATP producing (phosphorylating state). State 4 reflects the O_2 consumption after the mitochondria have already depleted all available ADP, reducing the respiration rate (non- phosphorylating state) (Nicholls and Ferguson, 2002). When the proton gradient is dissipated by the action of ATP synthase, due to ADP addition, there is an imbalance that drives the transfer of electrons through the respiratory chain, and therefore the oxygen consumption. Thus, the activation of the ATP synthase requires two factors: availability of ADP and high membrane potential (Nelson and Cox, 2008). In this context, the coupling of respiration is defined as the ability of mitochondria to generate energy (ATP) when exposed to ADP, linking the processes of oxidation and phosphorylation. The dissipation of the proton electrochemical gradient as a consequence of mitochondrial damage or increased permeability of the inner mitochondrial membrane uncouples electron transport from the ATP synthesis, leading to augmented oxygen consumption (increased respiration) with reduced formation of ATP (Nicholls and Ferguson, 2002).

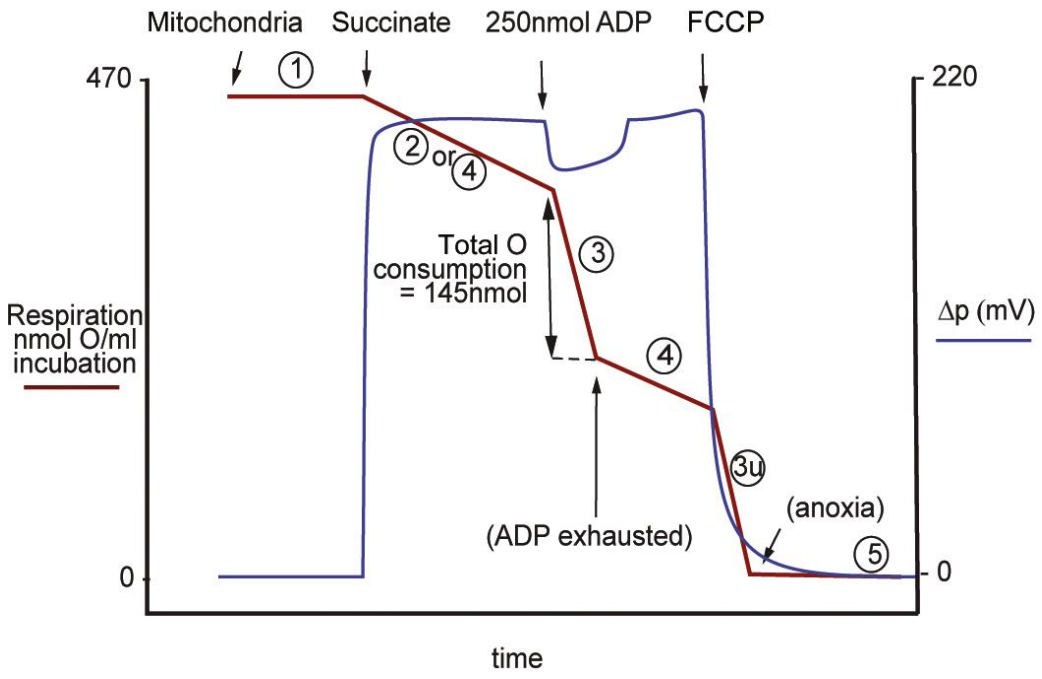

Figure 4. States of mitochondrial respiration (Nicholls and Ferguson, 2002).

1.5 Role of Mitochondria in Ca^{2+} Homeostasis

Maintenance of calcium homeostasis is another important function performed by mitochondria since calcium coordinates a wide range of processes in the cell (Figueira et al., 2013; Rizzuto et al., 2012). When fully functional, this organelle has the ability to keep the Ca^{2+} intracellular concentration by capturing the Ca^{2+} present in the cytosol or releasing the stored ion (Figueira et al., 2013; Rizzuto et al., 2012). In the mitochondrial matrix, Ca^{2+} can form inactive precipitates with phosphate, which makes the mitochondria capable of accumulating high Ca^{2+}concentrations (Starkov, 2010). In this purpose, Ca^{2+} needs to be captured or released by mitochondria. To access the intermembrane space or be released back into the cytosol, Ca^{2+} uses the voltage-dependent ion channel (VDAC) located in the outer mitochondrial membrane. Once in the intermembrane space, Ca^{2+} enters into the mitochondria by

a uniport system (MCU) and is released from the matrix by the exchangers Na^+/Ca^{2+} (mNCX) and H^+/Ca^{2+} (mHCX) (Bernardi and von Stockum, 2012; Rizzuto et al., 2012) (Figure 5).

Many conditions can lead to Ca^{2+} overload in the cell as ischemia/reperfusion, hypoglycemia, as well as NMDA activation in the central nervous system. Such conditions lead to activation of phospholipase A2, a lytic enzyme, besides proteases and endonucleases, resulting in cell death (Orrenius et al., 2003). Under conditions of Ca^{2+} overload, the mitochondrion becomes the major organelle responsible for capturing excess of cytosolic Ca^{2+} to maintain cell function (Figueira et al., 2013; Rizzuto et al., 2012). On the other hand, a high mitochondrial uptake of Ca^{2+}, beyond its capacity, may lead to a condition known as permeability transition as a result of opening of a pore in the inner mitochondrial membrane (Adam-Vizi and Starkov, 2010; Starkov, 2010; Zoratti and Szabo, 1995). The mitochondrial permeability transition pore (mPTP) is formed by proteins present in the mitochondrial matrix and in the inner mitochondrial membrane (Starkov, 2010), however its precise composition is still under investigation. The matrix protein cyclophilin D (Cyp D) seems to be a key component for the occurrence of the mPTP (Figure 5). In addition, ADP is a potent inhibitor of mPTP through its binding to the adenine nucleotide translocator (ANT), another mitochondrial carrier involved in the mPTP formation (Rottenberg and Marbach, 1989; Saito and Castilho, 2010).

The permanent opening of the mPTP results in depolarization, impairment of ATP synthesis, mitochondrial swelling and rupture of the outer membrane provoking the loss of small molecules (Ca^{2+}, Mg^{2+}, reduced glutathione (GSH), NADH and NADPH) to the cytosol, as well as the release of apoptotic factors as cytochrome *c* (Penna et al., 2013; Rasola and Bernardi, 2011).

The mPTP is inhibited by cyclosporin A (CsA), a known peptide that binds Cyp D (Bernardi and von Stockum, 2012). CsA has a protective effect in models of ischemia (Nieminen et al., 1996; Schinder et al., 1996; Uchino et al., 1998), suggesting the importance of mPTP in the pathogenesis of

ischemic damage. Moreover, there is growing body of evidence indicating that a disturbance in Ca^{2+} homeostasis and the incidence of mPTP contribute to neurological dysfunction in a variety of diseases which are associated with neurodegeneration (Figueira et al., 2013; Murphy et al., 1999) and myocardial infarct/reperfusion (Davidson et al., 2012; Penna et al., 2013).

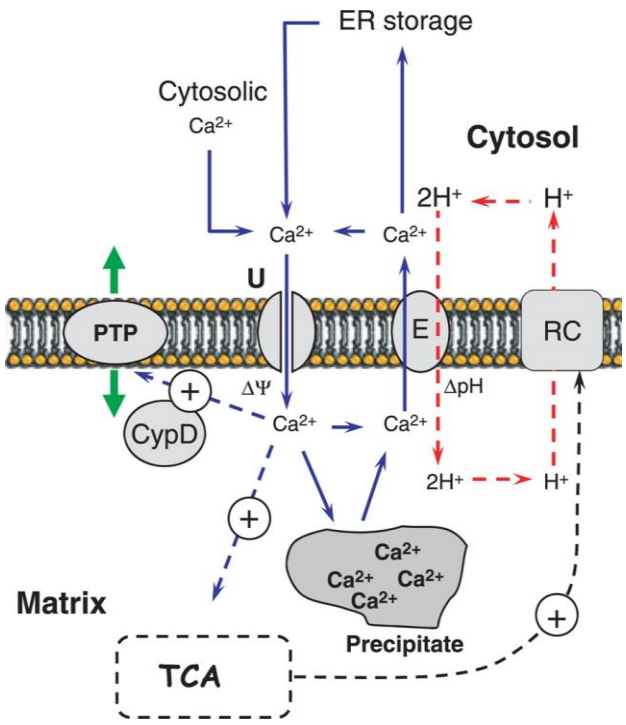

Figure 5. Standard model of mitochondrial Ca^{2+} handling. Mitochondrial Ca^{2+} carrier (Ca^{2+} uniporter, U); inner mitochondrial membrane (IM); $Ca^{2+}/2H^{+}$ exchanger (E); cyclophilin D (CypD); respiratory chain (RC); tricarboxylic acid cycle (TCA) (Starkov, 2010).

1.6. Mitochondria and free radicals

During the oxidative phosphorylation process, electrons can escape from the electron transport chain and partially reduce oxygen giving rise to reactive oxygen species (ROS). The mitochondrial ETS generates ROS mainly at complexes III and I of the respiratory chain, but also at complex II, ETF and ETF dehydrogenase, the pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase complex (Quinlan, 2011).

A free radical is any chemical species able to exist independently and containing one or more unpaired electrons (Halliwell, 2006). Under physiological conditions of aerobic cellular metabolism, molecular oxygen (O_2) is tetravalent reduced, incorporating four electrons resulting in the formation of water (H_2O). However, approximately 5 % of the oxygen used in the mitochondrial respiratory chain are not completely reduced to water giving rise to ROS such as superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). Besides ROS, there are still the reactive nitrogen species (RNS), represented mainly by nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$).

The ROS and RNS occur both in physiological or pathological conditions. Physiologically, these reactive species participate in different cells functions being released by neutrophils and involved in cell signaling processes and synthesis and regulation of some proteins (Delanty and Dichter, 1998; Halliwell and Gutteridge, 2007). However, when formed in excess, these highly reactive species have the potential to oxidize molecules promoting lipid peroxidation, as well as protein oxidative damage, leading to inactivation and subsequent modification of their function. Moreover, they can induce DNA and RNA oxidation leading to somatic mutations and affect transcription process (Delanty and Dichter, 1998; Halliwell and Whiteman, 2004).

1.6.1 Antioxidant defenses

In order to prevent the damaging effects of reactive species, the cells hold efficient mechanisms as antioxidant enzymes and non-enzymatic antioxidants. Although differing in composition, antioxidant defenses are widely distributed in the body (Halliwell and Gutteridge, 2007) and comprise:

• Enzymes responsible for the removal of reactive species such as superoxide dismutase, catalase and glutathione peroxidase;

• Proteins that minimize the availability of pro-oxidant molecules such as iron and copper ions, by binding to these ions;

• Proteins that protect biomolecules from oxidative damage;

• Low molecular weight agents that trap ROS and RNS such as glutathione, alpha-tocopherol, ascorbic acid and bilirubin.

1.6.2 Oxidative Stress

Therefore, healthy bodies produce reactive species that when in excess are controlled by antioxidant defense systems. However, in certain pathological conditions an imbalance between the production of oxidants and antioxidant defenses can occur, favoring the occurrence of oxidative stress. In this scenario, the term "oxidative stress" refers to conditions in which the generation of reactive species exceeds the capacity of the available antioxidant defenses. Such conditions may result either from a decrease in antioxidant defenses or an increased production of pro-oxidants (Halliwell, 2006).

The oxidative stress can promote cell adaptation, damage or death:

• Adaptation: the cells can tolerate a moderate oxidative stress level, which often results in an increased synthesis of antioxidants defenses in order to restore the balance oxidant/antioxidant. Nevertheless, oxidative stress does not always have to involve increased antioxidant defenses.

• Damage: oxidative stress can damage molecular targets as DNA, proteins, carbohydrates and lipids impairing their physiological functions (Halliwell and Gutteridge, 2007). However, the injury can be reverted and does not lead to cell death.

• Cell death: can occur either by necrosis or by apoptosis. In necrotic cell death, the cell swells and ruptures, releasing their contents into the extracellular environment. The release of cell components may affect adjacent cells, even leading them to an oxidative stress condition. In apoptotic cell death, a pathway is activated and the cell death occurs in a more controlled way.

2. Objective

The aim of the present work was to clarify the mechanisms that underlie the toxic effects of the fatty acids and derivative compounds that accumulate in MCAD, LCHAD and MTP deficiencies, especially the influence of these metabolites on redox and energy homeostasis in brain structures and heart of young rats, as well as to evaluate ROS production and cell response to metabolic stress in skin fibroblasts from patients affected by MCAD and MTP deficiencies.

2.1. Specific objectives

1. Investigate the role of the medium-chain acylcarnitines that most accumulate in MCAD deficient patients on important parameters of redox homeostasis in rat brain (CHAPTER I);
2. Investigate superoxide production and cell death in cultured skin fibroblasts from normal human dermal fibroblasts (controls) and patients affected by MCAD and MTP deficiencies under metabolic stress conditions (CHAPTER II);
3. Evaluate the effects of long-chain 3-hydroxylated fatty acids that accumulates in LCHAD and MTP deficiencies on important parameters of mitochondrial bioenergetics in the rat heart (CHAPTER III);
4. Investigate the effect of long-chain 3-hydroxylated fatty acids that accumulates in LCHAD and MTP deficiencies on important parameters of mitochondrial homeostasis in cerebral cortex mitochondrial preparations challenged by Ca^{2+} (CHAPTER IV);
5. Investigate the role of long-chain 3-hydroxylated fatty acids that accumulates in LCHAD and MTP deficiencies on important parameters of redox homeostasis in rat heart (CHAPTER V).

SECTION II

CHAPTER I

Disruption of redox homeostasis in cerebral cortex of developing rats by acylcarnitines accumulating in medium-chain acyl-CoA dehydrogenase deficiency

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Scientific article published in

International Journal of Developmental Neuroscience 30(5):383-90, 2012

Int. J. Devl Neuroscience 30 (2012) 383–390

Contents lists available at SciVerse ScienceDirect

International Journal of Developmental Neuroscience

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

Disruption of redox homeostasis in cerebral cortex of developing rats by acylcarnitines accumulating in medium-chain acyl-CoA dehydrogenase deficiency

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ARTICLE INFO

Article history:
Received 22 January 2012
Received in revised form 7 March 2012
Accepted 17 March 2012

Keywords:
MCAD deficiency
Acylcarnitines
Lipid oxidative damage
Protein oxidative damage
Antioxidant defenses
Rat brain

ABSTRACT

Medium-chain fatty acids and acylcarnitines accumulate in medium-chain acyl-CoA dehydrogenase deficiency (MCADD), the most frequent fatty acid oxidation defect clinically characterized by episodic crises with vomiting, seizures and coma. Considering that the pathophysiology of the neurological symptoms observed in MCADD is poorly known and, to our knowledge, there is no report on the involvement of acylcarnitines in the brain damage presented by the affected patients, the objective of the present study was to investigate the *in vitro* effects of hexanoylcarnitine (HC), octanoylcarnitine, decanoylcarnitine (DC) and cis-4-decenoylcarnitine (cDC) at concentrations varying from 0.01 to 1.0 mM on important oxidative stress parameters in cerebral cortex of young rats. HC, DC and cDC significantly induced lipid peroxidation, as determined by increased thiobarbituric acid-reactive substances (TBA-RS) values. In addition, carbonyl formation was significantly augmented and sulfhydryl content diminished by DC, reflecting induction of protein oxidative damage. HC, DC and cDC also decreased glutathione (GSH) levels, the most important brain antioxidant defense. Furthermore, DC-induced elevation of TBA-RS values and decrease of GSH levels were prevented by the free radical scavengers melatonin and α-tocopherol, indicating the involvement of reactive oxygen species in these effects. We also found that L-carnitine itself did not induce lipid and protein oxidative damage, neither reduced the antioxidant defenses. Our present data show that the major medium-chain acylcarnitines accumulating in MCADD elicit oxidative stress in rat brain. It is therefore presumed that these compounds may be involved to a certain extent in the pathogenesis of the neurologic dysfunction of MCADD.

1. Introduction

Medium-chain acyl-CoA dehydrogenase deficiency (MCADD), the most frequent inherited disorder of fatty acid oxidation (FAO), is biochemically characterized by accumulation of medium-chain fatty acids (MCFA) and their L-carnitine esters (Rinaldo et al., 2002) in tissues and body fluids. MCAD-deficient patients present characteristic episodes of acute decompensation under metabolic stress usually due to prolonged fasting or following infections. During these crises of metabolic decompensation, when energy from FAO is most needed, the affected individuals manifest severe symptoms, such as vomiting, hypotonia, seizures and coma that may be associated with hypoglycemia and hyperammonemia, and marked increases of the free MCFA hexanoic, octanoic, decanoic and cis-4-decenoic acids, as well as their L-carnitine conjugates hexanoylcarnitine (HC), octanoylcarnitine (OC), decanoylcarnitine (DC) and cis-4-decenoylcarnitine (cDC) (Roe and Ding, 2001). The most dramatic outcome is sudden unexplained death that occurs in approximately 15–20% of children with MCADD. Furthermore, approximately 20% of unselected patients diagnosed during crises before introduction of expanded newborn screening in the general population present sustained neurological damage (Pollitt and Leonard, 1998; Wilcken et al., 1994, 2007). However, recent data from affected children diagnosed in the neonatal period indicate that the risk of intellectual deficit or other morbidities is extremely small, probably because of better emergency treatment, which usually prevents crises of metabolic decompensation (Wilcken, 2010).

Abbreviations: HA, hexanoic acid; HC, hexanoylcarnitine; HG, hexanoylglycine; OC, octanoylcarnitine; DC, decanoylcarnitine; cDC, cis-4-decenoylcarnitine; MCAC, medium-chain acylcarnitines.

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0736-5748/$36.00 © 2012 ISDN. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijdevneu.2012.03.238

Despite the high prevalence of MCADD in the population, which is similar to phenylketonuria (1:10,000 newborns), the exact underlying mechanisms responsible for the neuropathology of MCADD are still poorly established. In this scenario, the rapid deterioration of the clinical features in affected patients generally precedes the development of hypoglycemia, suggesting a potential toxic role for the accumulating MCFA and their derivatives in the pathogenesis of MCADD, in addition to hypoketotic hypoglycemia (Gregersen et al., 2008). This is consistent with the observations that the MCFA accumulating in this disease disturb mitochondrial homeostasis by inhibiting the citric acid cycle, the respiratory chain flow, Na^+ K^+ ATPase and mitochondrial creatine kinase activities, besides inducing oxidative stress in rat brain (de Assis et al., 2006; Reis de Assis et al., 2004; Schuck et al., 2007, 2009). However, regarding to the medium-chain acylcarnitines (MCAC), the only study reporting their effects on metabolism revealed that these compounds do not alter the respiratory chain complex activities measured in submitochondrial particles, neither commercially purified α-ketoglutarate dehydrogenase or pyruvate dehydrogenase activities prepared from bovine and porcine heart (Sauer et al., 2008).

Since oxidative damage has been associated with common neurodegenerative diseases and considered an important pathogenic mechanism of these disorders (Behl and Moosmann, 2002; Bogdanov et al., 2001; Perez-Severiano et al., 2000), the aim of the present study was to investigate the role of the MCAC that most accumulate in MCADD on important parameters of redox homeostasis in rat brain. We tested the *in vitro* effects of HC, OC, DC and cDC at concentrations varying from 0.01 to 1.0 mM on thiobarbituric acid-reactive substances (TBA-RS) values (lipid oxidation), carbonyl formation and sulfhydryl oxidation (protein oxidation) and reduced glutathione (GSH) levels (antioxidant defenses) in cerebral cortex of developing rats (Halliwell and Gutteridge, 2007). We also tested the effects of L-carnitine on some parameters of oxidative stress and the role of antioxidants on the alterations elicited by the MCAC on TBA-RS and GSH levels.

2. Material and methods

2.1. Animals and reagents

Seventy two thirty-day-old male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, were used (6 animals in each experiment). The animals were maintained on a 12:12 h light/dark cycle (lights on 07.00–19.00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil and followed the Principles of Laboratory Animal Care (NIH publication 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma (St. Louis, MO, USA), except for the acylcarnitines and hexanoylglycine (99% purity) that were prepared by Dr. Ernesto Brunet, Madrid, Spain. The metabolites used were dissolved on the day of the experiments in the incubation medium used for each technique and had their pH adjusted to 7.4. The final concentrations of these metabolites in the medium, as well as L-carnitine that was used in some experiments, ranged from 0.01 to 1 mM.

2.2. Tissue preparation and incubation

On the day of the experiments the animals were sacrificed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum and striatum were discarded, and the cerebral cortex was peeled away from the subcortical structures, weighed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 × *g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl at 37 °C for 1 h with the acylcarnitines (HC, OC, DC, cDC). We also utilized hexanoic acid (HA), hexanoylglycine (HG) and L-carnitine in some assays. Controls did not contain any of these metabolites in the incubation medium.

In some experiments, antioxidants were co-incubated with supernatants at the following final concentrations: 10 μM Trolox (TRO, soluble α-tocoferol), 1500 μM melatonin (MEL), 500 μM reduced glutathione (GSH), 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) and the combination of SOD plus CAT (100 mU each). The chosen concentrations of the antioxidants were those capable to efficiently scavenge free radicals (Halliwell and Gutteridge, 2007; Leipnitz et al., 2011).

We always carried out parallel experiments with blanks (controls) in the presence or absence of the tested metabolites and without cortical supernatants in order to detect artifacts caused by the MCAC in the assays.

2.3. Determination of thiobarbituric acid-reactive substances (TBA-RS) levels

TBA-RS levels were determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of pre-incubated cerebral cortex supernatants and centrifuged at 750 × *g* for 10 min. Three hundred microliters of the supernatant (containing approximately 0.3 mg of protein) were transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulfate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol of TBA-RS/mg protein.

2.4. Determination of protein carbonyl formation

Protein carbonyl content, a marker of oxidized proteins, was measured spectrophotometrically according to Reznick and Packer (1994). One hundred microliters of the aliquots from the incubation (containing approximately 0.3 mg of protein) were treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank control) and left in the dark for 1 h. Samples were then precipitated with 500 μL 20% TCA and centrifuged for 5 min at 10,000 × *g*. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and re-dissolved in 550 μL 6 M guanidine prepared in 2.5 N HCl. Then, the tubes were incubated at 37 °C for 5 min to assure the complete dissolution of the pellet and the resulting sample was determined at 365 nm. The difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl content. The results were calculated as nmol of carbonyl groups/mg of protein, using the extinction coefficient of 22,000 × 106 nmol/mL for aliphatic hydrazones.

2.5. Determination of sulfhydryl (thiol) group content

This assay is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 0.1 mM DTNB was added to pre-treated cortical supernatants (containing approximately 0.3 mg of protein). This was followed by 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. The protein-bound sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

2.6. Determination of reduced glutathione (GSH) concentrations

Reduced glutathione (GSH) concentrations were measured according to Browne and Armstrong (1998). Metaphosphoric acid solution (1.8%) was added to the pre-treated cerebral cortex supernatants (1:1, v/v) to precipitate proteins and centrifuged for 10 min at 7000 × *g*. Then, the supernatants (containing approximately 0.3 mg of protein) were diluted (1:20, v/v) in 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation were incubated with an equal volume of *o*-phthaldialdehyde (1 mg/mL methanol) at room temperature during 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.01–1 mM) and the concentrations were calculated as nmol/mg protein.

2.7. Protein determination

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

2.8. Statistical analysis

Results are presented as mean ± standard deviation of the mean. Assays were performed in triplicate and the mean was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the *post hoc* Duncan multiple range test when *F* was significant. Pearson's linear coefficient of correlation and linear regression analysis were also used in some experiments. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

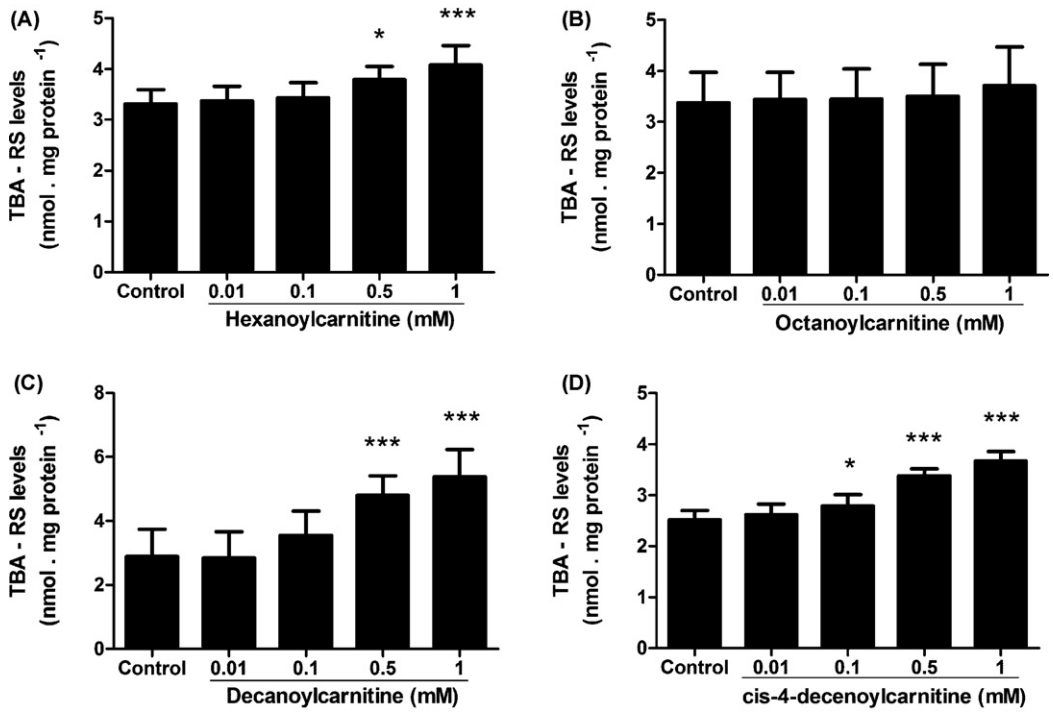

Fig. 1. *In vitro* effect of hexanoylcarnitine (HC) (A), octanoylcarnitine (OC) (B), decanoylcarnitine (DC) (C) and cis-4-decenoylcarnitine (cDC) (D) on thiobarbituric acid-reactive substances (TBA-RS) levels in rat cerebral cortex. Values are means ± standard deviation of six independent experiments (animals) performed in triplicate. The parameter was calculated as nmol/mg protein. Controls did not contain the tested acylcarnitines. $^{*}P < 0.05$, $^{***}P < 0.001$, relatively to controls (Duncan multiple range test).

3. Results

3.1. Lipid peroxidation is induced by hexanoylcarnitine (HC), decanoylcarnitine (DC) and cis-4-decenoylcarnitine (cDC) in rat cerebral cortex

We initially tested the *in vitro* influence of HC, OC, DC and cDC on TBA-RS levels, a lipid oxidation marker, in cerebral cortex. Fig. 1 shows that HC (A), DC (C) and cDC (D) significantly increased TBA-RS levels relatively to controls [HC: $P < 0.001$; DC: $P < 0.001$; cDC: $P < 0.001$] in a dose-dependent manner [HC: $\beta = 0.718$; $P < 0.001$; DC: $\beta = 0.914$; $P < 0.001$; cDC: $\beta = 0.908$; $P < 0.001$]. Furthermore, it can be observed in the figure that the strongest effect was achieved with DC (up to 86%), followed by cDC (up to 46%) and by HC (up to 23%).

3.2. Decanoylcarnitine induces protein oxidative damage in rat cerebral cortex

Next, we evaluated the effect of HC, OC, DC, cDC on carbonyl formation and sulfhydryl oxidation in order to evaluate protein oxidation parameters. We found that only DC significantly increased protein carbonyl formation (up to 40%) [$P < 0.001$], as compared to controls (Fig. 2C). DC also decreased sulfhydryl content (up to 35%) [$P < 0.001$] in a dose-dependent way [DC: $\beta = -0.742$; $P < 0.001$] relatively to controls (Fig. 3C). In contrast, the other acylcarnitines tested had no effect on these parameters.

3.3. GSH concentrations are decreased by hexanoylcarnitine, decanoylcarnitine and cis-4-decenoylcarnitine in rat cerebral cortex

The *in vitro* effect of HC, OC, DC and cDC on the concentrations of GSH, the most important brain antioxidant defense, was then investigated. We found that HC [$P < 0.01$], DC [$P < 0.001$] and cDC [$P < 0.01$] significantly decreased GSH levels by up to 18%, 50% and 20%, respectively, relatively to controls (Fig. 4). Furthermore, DC effect was dose-dependent [DC: $\beta = -0.777$; $P < 0.001$]. It can be also seen in Fig. 4B that OC did not alter GSH concentrations.

3.4. Antioxidants prevent decanoylcarnitine (DC)-increase of lipid peroxidation and decrease of GSH levels

We then evaluated the role of the antioxidants GSH (500 μM), TRO (10 μM), MEL (1500 μM), L-NAME (500 μM) and SOD plus CAT (100 mU each) on the effects elicited by DC on TBA-RS and GSH concentrations. We observed that TRO and MEL completely prevented the DC-increase of TBA-RS values, whereas GSH, L-NAME and SOD plus CAT did not change the increase of TBA-RS provoked by DC [$P < 0.001$]. Furthermore, TRO attenuated, whereas MEL totally prevented DC-decrease of GSH levels, whereas L-NAME and SOD plus CAT did not change the reduction of GSH concentrations induced by DC [$P < 0.001$] (Fig. 5). These data indicate that the increased lipid peroxidation and decreased GSH concentrations provoked by DC were mediated through reactive oxygen species, and probably by peroxyl and hydroxyl radicals that are mainly scavenged by TRO and MEL. Since L-NAME did not have any effect on the alterations provoked by DC, it is presumed that nitrogen reactive species were not involved in its effects.

3.5. Decanoylcarnitine-induced reduction of GSH concentrations and increase of TBA-RS values were inversely correlated

We also found that DC-induced reduction of GSH concentrations was inversely correlated with TBA-RS values [$r = -0.811$; $P < 0.001$] (Fig. 6), implying that both effects were probably mediated by reactive species in a similar fashion and that free radical-inducing lipid peroxidation was probably responsible for the decrease of the antioxidant defenses caused by this acylcarnitine.

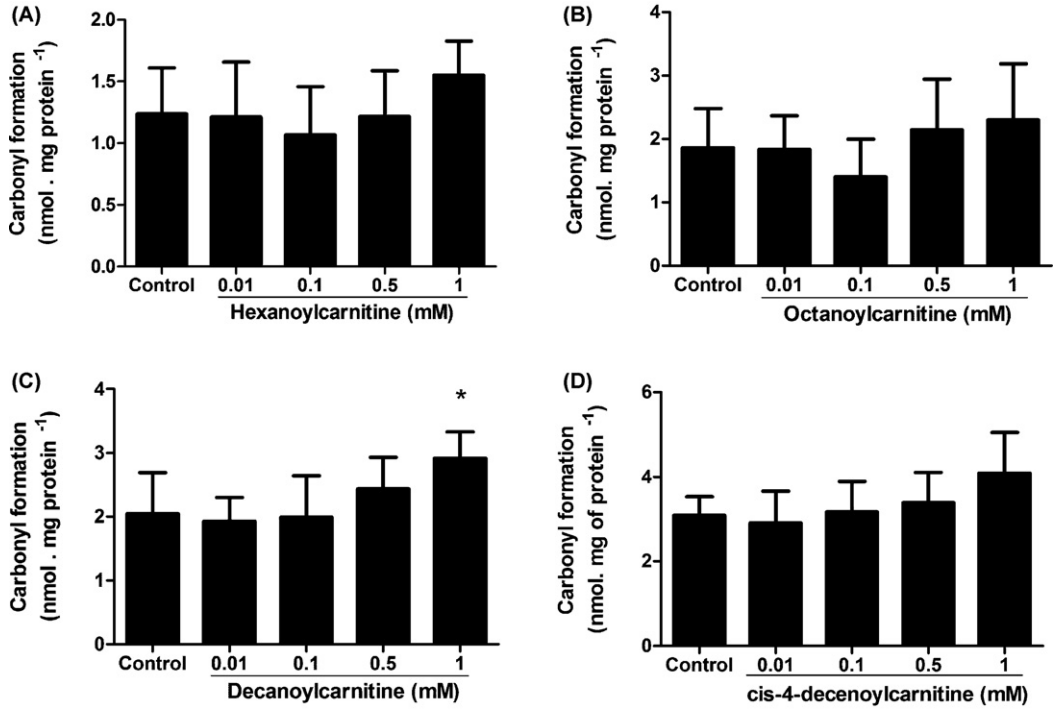

Fig. 2. *In vitro* effect of hexanoylcarnitine (HC) (A), octanoylcarnitine (OC) (B), decanoylcarnitine (DC) (C) and cis-4-decenoylcarnitine (cDC) (D) on carbonyl formation in rat cerebral cortex. Values are means ± standard deviation of six independent experiments (animals) performed in triplicate. The parameter was calculated as nmol/mg protein. Controls did not contain the tested acylcarnitines. $^{*}P < 0.05$, relatively to controls (Duncan multiple range test).

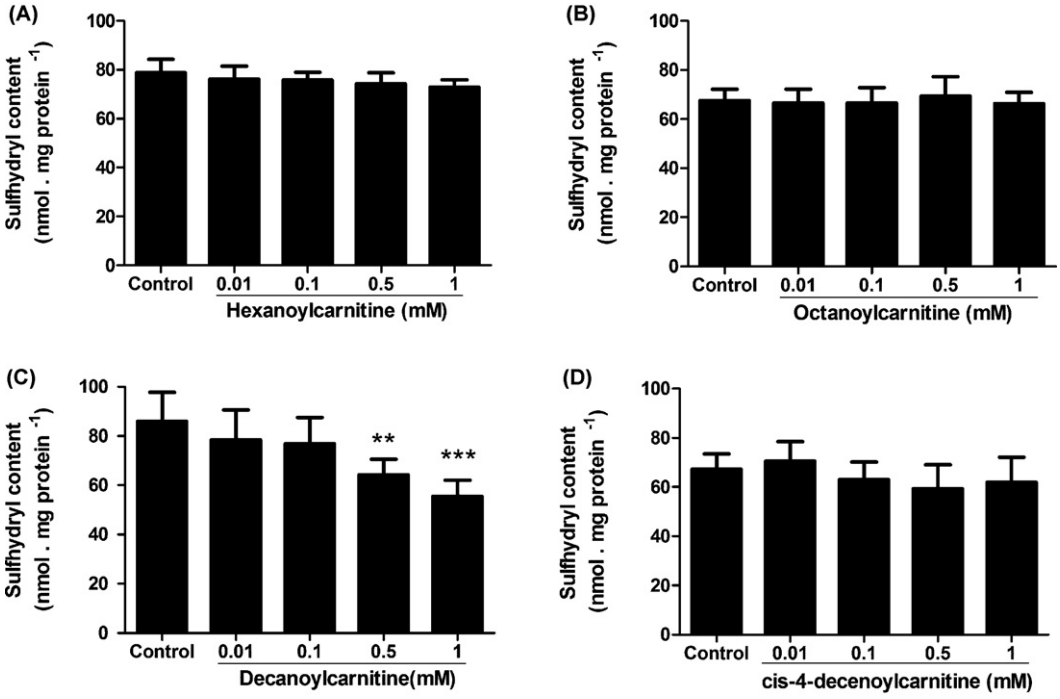

Fig. 3. *In vitro* effect of hexanoylcarnitine (HC) (A), octanoylcarnitine (OC) (B), decanoylcarnitine (DC) (C) and cis-4-decenoylcarnitine (cDC) (D) on sulfhydryl content in rat cerebral cortex. Values are means ± standard deviation of six independent experiments (animals) performed in triplicate. The parameter was calculated as nmol/mg protein. Controls did not contain the tested acylcarnitines. $^{**}P < 0.01$, $^{***}P < 0.001$, relatively to controls (Duncan multiple range test).

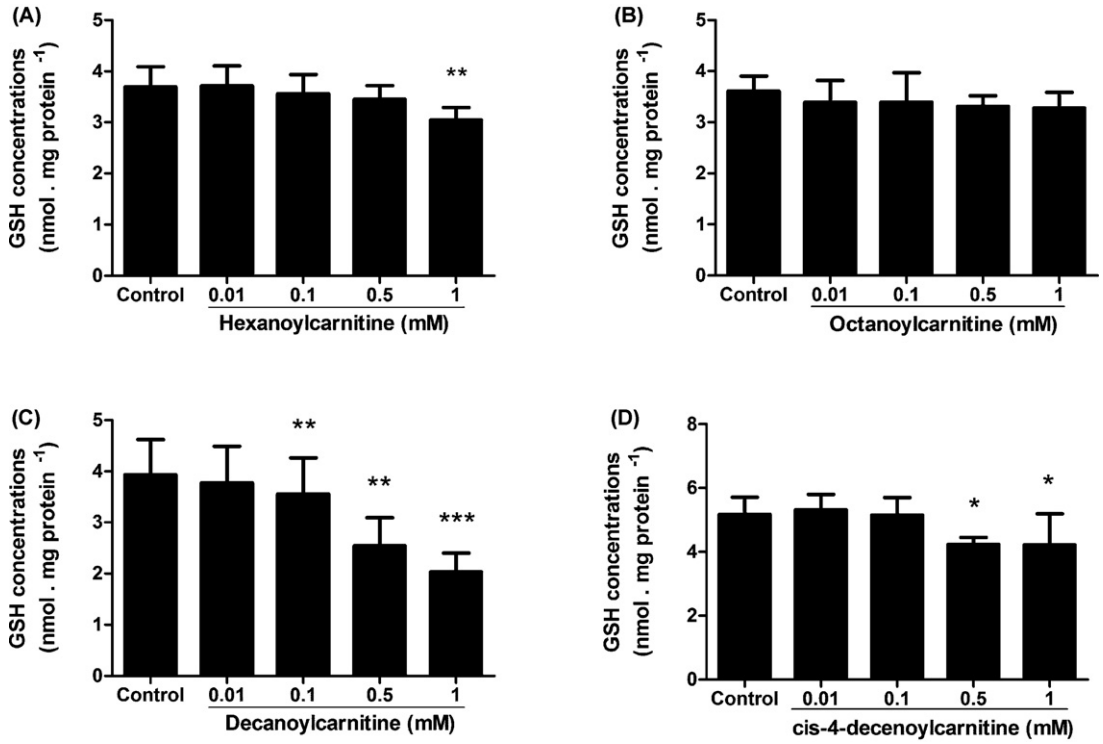

Fig. 4. *In vitro* effect of hexanoylcarnitine (HC) (A), octanoylcarnitine (OC) (B), decanoylcarnitine (DC) (C) and cis-4-decenoylcarnitine (cDC) (D) on reduced glutathione (GSH) levels in rat cerebral cortex. Values are means ± standard deviation of six independent experiments (animals) performed in triplicate. The parameter was calculated as nmol/mg protein. Controls did not contain the tested acylcarnitines. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, relatively to controls (Duncan multiple range test).

3.6. Hexanoic acid (HA) and hexanoylglycine (HG) do not alter TBA-RS values and GSH concentrations in rat cerebral cortex

We also tested the effect of HA and its L-carnitine (HC) and glycine (HG) derivatives on TBA-RS values, carbonyl formation, sulfhydryl content and GSH concentrations in order to compare their effects on these parameters. We can observe in Fig. 7 that HC significantly increased TBA-RS concentration (A) [$P < 0.001$], decreased GSH concentrations (D) [$P < 0.01$], with no effect on carbonyl formation (B) and sulfhydryl content (C). Furthermore, HA and HG did not alter any of these parameters.

3.7. L-Carnitine does not change TBA-RS values, sulfhydryl content and GSH concentrations

Considering that only HC increased malondialdehyde formation (TBA-RS levels) and decreased GSH concentrations, with no effect for HA and HC, it could be presumed that the L-carnitine component of the molecule was responsible for these effects. We therefore tested the effects of commercial L-carnitine at doses ranging from 0.1 to 1 mM on these parameters. We verified that L-carnitine does not alter TBA-RS values, sulfhydryl content and GSH concentrations (Table 1), implying a selective effect for HC.

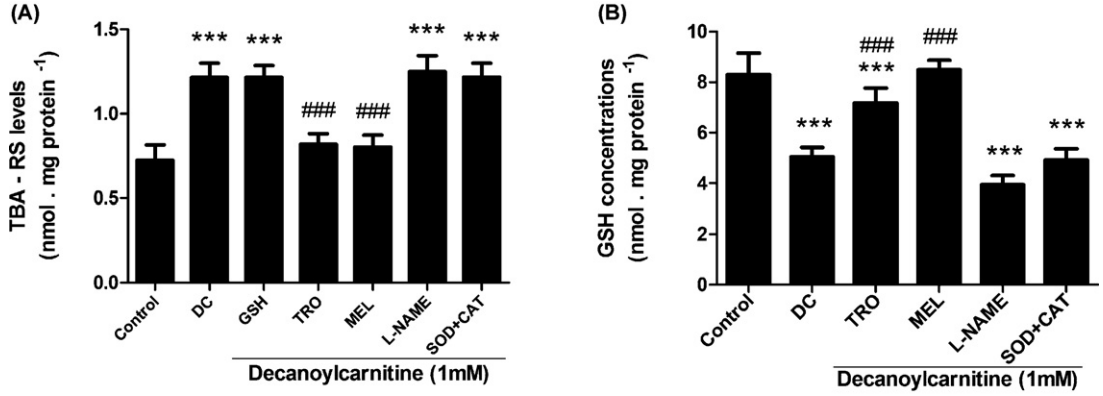

Fig. 5. *In vitro* effect of antioxidants on decanoylcarnitine (DC)-induced increase of lipid peroxidation (TBA-RS increase) (A) and reduction of GSH levels (B) in rat cerebral cortex. Cortical supernatants were co-incubated with 500 μM of reduced glutathione (GSH), 1500 μM of melatonin (MEL), 10 μM Trolox (TRO, soluble α-tocoferol), 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) or 100 mU superoxide dismutase plus 100 mU catalase (SOD plus CAT) and 1 mM DC during 1 h. Values are means ± standard deviation of six independent (animals) experiments performed in triplicate and are expressed nmol/mg protein. $^{***}P < 0.001$, compared to control; $^{\#\#\#}P < 0.001$, compared to 1 mM DC (Duncan multiple range test).

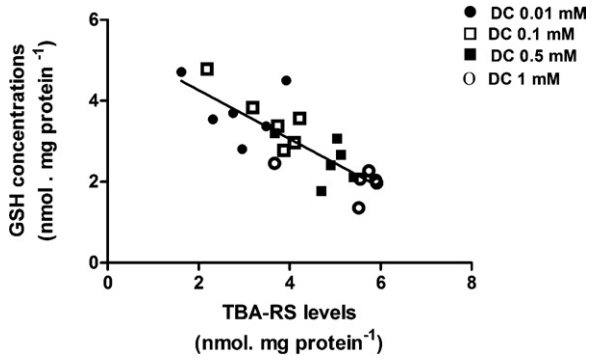

Fig. 6. Correlation between thiobarbituric acid-reactive substances (TBA-RS) and reduced glutathione (GSH) in rat cerebral cortex exposed to 0.01–1.0 mM decanoylcarnitine (DC). Values are expressed as nmol/mg protein.

Table 1
In vitro effect of L-carnitine (L-CAR) on thiobarbituric acid-reactive substances (TBA-RS) values, sulfhydryl content and reduced glutathione (GSH) concentrations in cerebral cortex from young rats.

	TBA-RS values	Sulfhydryl content	GSH concentrations
Control	2.96 ± 0.27	49.3 ± 10.8	2.37 ± 0.13
0.01 mM L-CAR	2.99 ± 0.33	48.9 ± 13.1	2.39 ± 0.31
0.1 mM L-CAR	2.98 ± 0.40	47.9 ± 7.8	2.32 ± 0.30
0.5 mM L-CAR	3.00 ± 0.30	47.3 ± 8.8	2.22 ± 0.33
1 mM L-CAR	3.19 ± 0.29	53.4 ± 24.4	2.22 ± 0.34

Values are expressed as mean ± standard deviation of six independent experiments (animals) performed in triplicate. Results were calculated as nmol/mg protein. No significant differences between groups were detected (one-way ANOVA).

4. Discussion

A variable degree of neurological dysfunction associated with cerebral MRI alterations can be observed in a considerable number of unselected MCADD patients diagnosed prior to the establishment of expanded neonatal mass screening (Mayatepek et al., 1997; Ruitenbeek et al., 1995; Schatz and Ensenauer, 2010; Scrace et al., 2008; Smith and Davis, 1993; Wilcken et al., 1994, 2007; Wilson et al., 1999). Brain abnormalities in these individuals seem to follow episodes of metabolic decompensation with coma, whose pathophysiology is still poorly known. In this context, the MCFA and their byproducts accumulating in this disorder have been tentatively associated with lethargy that may progress to coma and death in MCADD (Rinaldo et al., 2002). Previous experimental studies using rat brain revealed that the MCFA accumulating in this disease impair bioenergetics (de Assis et al., 2006; Reis de Assis et al., 2004) and induce oxidative stress (Schuck et al., 2007, 2009), indicating that these endogenous compounds accumulating in MCADD are potentially neurotoxic. However, another study failed to demonstrate alterations caused by the MCAC on various parameters of energy metabolism (Sauer et al., 2008), although to our knowledge nothing has been reported on the effects of these fatty acid carnitine derivatives on redox homeostasis.

Considering that oxidative stress has been considered an important underlying pathomechanism of common neurodegenerative disorders (Berg and Youdim, 2006; Mancuso et al., 2006; Stoy et al., 2005) and of some inborn errors of metabolism (Busanello et al., 2011; Ribas et al., 2011; Seminotti et al., 2011), in the present work we investigated the effects of MCAC on a large spectrum of relevant parameters of redox homeostasis in cerebral cortex from young rats. We used cerebral cortex in this investigation because brain abnormalities are seen in this structure in MCAD deficient patients.

We initially observed that HC, cDC and particularly DC provoked marked increases of TBA-RS levels that reflect the amount of malondialdehyde, a product of lipid peroxidation (Niki, 2009). Furthermore, the DC-induced lipid peroxidation was fully prevented by the antioxidants TRO and MEL, indicating that the lipid oxidative damage was mediated by reactive species. Considering that TRO, the hydrophilic analog of vitamin E, is an effective scavenger against peroxyl radicals (Halliwell and Gutteridge, 2007) and that hydroxyl and peroxyl radicals are mainly scavenged by MEL (Reiter, 1998), we presume that peroxyl and hydroxyl radicals were involved in

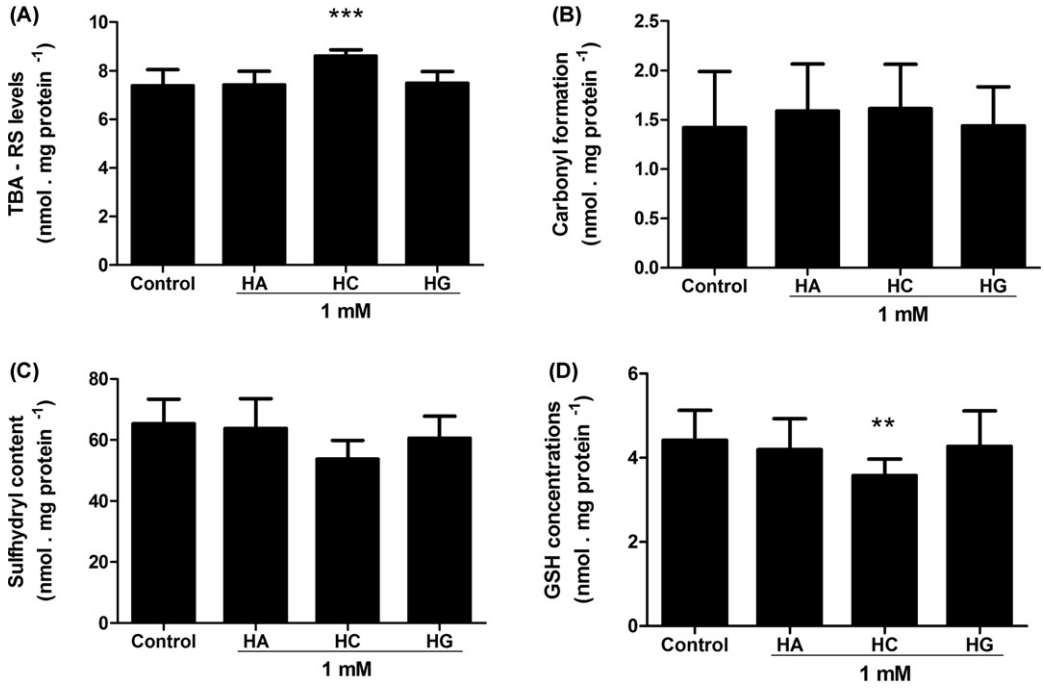

Fig. 7. *In vitro* effect of hexanoic acid (HA), hexanoylcarnitine (HC) and hexanoylglycine (HG) on thiobarbituric acid-reactive substances (TBA-RS) levels (A), carbonyl formation (B), sulfhydryl content (C) and reduced glutathione (GSH) levels (D) in rat cerebral cortex. Values are means ± standard deviation of six independent experiments (animals) performed in triplicate. The parameters were calculated as nmol/mg protein. $^{**}P < 0.01$, $^{***}P < 0.001$ relatively to controls (Duncan multiple range test).

the lipid peroxidation provoked by DC and the other acylcarnitines. On the other hand, it is unlikely that superoxide and hydrogen peroxide, scavenged by SOD plus CAT, respectively, and nitric oxide, whose synthesis is inhibited by L-NAME, mediated DC-induced *in vitro* lipid peroxidation since these antioxidants did not attenuate or block the induction of lipid oxidative damage provoked by DC.

Next, we investigated the effects of the MCAC on protein oxidation by measuring the carbonyl formed during incubation (Stadtman and Berlett, 1991) and sulfhydryl content. Protein carbonyl formation that results from an irreversible and non-enzymatic modification of proteins commonly due to increased reactive species generation (Stadtman and Levine, 2006) was increased by DC, indicating that this metabolite provoked protein oxidative damage. We cannot also exclude the possibility that aldehydes resulting from lipid peroxidation may have also induced carbonyl generation (Dalle-Done et al., 2003). Sulfhydryl content was also decreased by DC. As over two third of cellular sulfhydryl groups are protein-bound (Levine, 2002; Reznick and Packer, 1994), it is conceivable that these results allied to increased carbonyl formation reinforce the presumption that DC provokes protein oxidation. Furthermore, considering that protein oxidative damage usually leads to protein dysfunction, since many oxidized proteins lose their function (Dalle-Donne et al., 2009), our present results may possibly underlie, at least in part, the deleterious effects caused by DC.

We also verified that HC, DC and cDC markedly decreased brain GSH concentrations. Since GSH is generally used to evaluate the capacity of a tissue to prevent and respond to damage associated to the free radical processes (Evelson et al., 2001; Ferretti and Bacchetti, 2011; Halliwell and Gutteridge, 1999; Lissi et al., 1995), it may be concluded that the rat cortical antioxidant defenses were severely compromised by the MCAC and especially by DC, which exerted the most prominent effect. Similarly to the TBA-RS values, MEL and TRO, but not SOD plus CAT or L-NAME, were able to prevent DC-induced decrease of GSH levels in cerebral cortex, reinforcing the role of the highly toxic hydroxyl radical, which is the principal species scavenged by MEL (Ferretti and Bacchetti, 2011), and peroxyl radicals, which are scavenged by TRO, in the reduction of GSH concentrations provoked by DC. Therefore, although we cannot at the present establish the exact signal transduction pathways by which DC and the other MCAC induced lipid and protein oxidative damage in cerebral cortex, it may be presumed that free radicals elicited by these fatty acids could initiate the classical cascades leading to pro-oxidant effects. In this context we found a strong inverse correlation between TBA-RS increase and GSH decrease caused by DC, indicating that the decrease of the brain antioxidant defenses and the induction of lipid peroxidation are dependent effects.

We should also emphasize that the brain has a low capacity to react against free radicals because of its low antioxidant defenses compared to other tissues (Karelson et al., 2001), a fact that makes this tissue more vulnerable to increased reactive species. In fact, oxidative stress has been implicated in the pathophysiology of common neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, as well as in epileptic seizures and demyelination (Karelson et al., 2001; Mayatepek et al., 1997; Ruitenbeek et al., 1995; Smith and Davis, 1993).

Although we cannot at the present establish the mechanisms by which the MCAC elicited oxidative stress, these effects were not due to the carnitine component of their molecules since L-carnitine itself under identical conditions of the assays and at doses as high as 1 mM did not alter any of the parameters evaluated. Furthermore, our results showing that HA and HG did not induce lipid (TBA-RS increase) and protein (sulfhydryl decrease) oxidative damage or decreased the brain antioxidant defenses (GSH reduction), in contrast to HC that provoked these effects, indicate that carnitine derivatives of MCFA may be more toxic than the free fatty acids accumulating in MCADD.

We cannot also establish at the present the pathophysiological significance of our present data since to the best of our knowledge the brain and CSF concentrations of the MCAC in MCAD-deficient patients are unknown. However, we emphasize that DC and cDC provoked the most prominent alterations of the oxidative stress parameters (lipid peroxidation and reduction of antioxidant defenses) at 100 μM, which is probably a supraphysiological concentration. On the other hand, although plasma MCAC levels encountered in MCADD affected patients are much lower, we emphasize that during crises of acute encephalopathy the free MCFA concentrations from which the MCAC originate may reach in blood similar or even higher concentrations (100–400 μM) (Costa et al., 1998; Martínez et al., 1997) in these patients and that L-carnitine easily cross the blood–brain barrier (Jones et al., 2010; Méndez-Cuesta et al., 2011; Roe and Ding, 2001). Moreover, the enzymes of fatty acid oxidation are expressed in the neural cells (Tyni et al., 2004) and it has been observed that during fasting acylcarnitines and especially short-chain acylcarnitines increase in brain of normal rats at least 3-fold reaching levels of 160 μM (Murakami et al., 1997). It is therefore feasible that the brain concentrations of the accumulating acylcarnitines significantly increase in this disorder during metabolic crises potentially causing deleterious effects.

5. Conclusion

In conclusion, we report for the first time that the MCAC accumulating in MCADD provoke oxidative stress in cerebral cortex of developing rats probably at supraphysiological concentrations. Considering previous data showing that the free MCFA, from which the acylcarnitines originate and that appear in high concentrations in this disorder induce reactive species generation and oxidative damage in the brain (Schuck et al., 2007, 2009), it is tempting to speculate that excessive free radicals might contribute, at least in part, to the neuropathology of this disorder, especially during episodes of metabolic stress in which the tissue concentrations of the accumulating metabolites increase due to accelerated catabolism.

Acknowledgments

This work was supported by grants from CNPq, PRONEX II, FAPERGS, PROPESQ/UFRGS, and FINEP research grant Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00, Instituto Nacional de Ciência e Tecnologia, Excitotoxicidade e Neuroproteção (INCT-EN) and Programa Nacional de Pós-doutorado-CAPES.

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CHAPTER II

Increased levels of superoxide and cell death in skin fibroblasts from patients affected by medium-chain acyl-CoA dehydrogenase and mitochondrial trifunctional protein deficiencies

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1. Material and Methods

1.1 Patients and control samples

Cultured human skin fibroblast from four patients with MCADD, four unrelated patients with MTPD and from four healthy controls were included in the study. MCAD deficient cells with missense mutations were: MCADD1 - homozygous for the common c.985A>G mutation and MCADD2 - compound heterozygous for the common c.985A>G and for the missense mutation c.199C>T: ACADM c.[199T>C] + [985G>A] p. [Tyr42His] + [Lys329Glu]. MCAD deficient cells affected by null mutations were MCADD3 and MCADD4: ACADM [null] + [null]. MTPD deficient cells affected by mild missense mutations were: MTPD1 - HADHA c.[731C>T]+[IVS9-2A>G]/ p.[Ala244Ser]+[null]; and MTPD2 - HADHB c.[362T>C]+[696-697ins4]/p.[Leu121Pro]+[null]. MTPD patients affected by severe missense mutations were: MTPD3 - HADHA c.1528 heterozygous G>C/G, c.1678 C>T/C; and MTPD4 - HADHB c.520-521 ins AT ex 8 c.1376 C>A/C ala gln ex 15. Results were displayed for individual controls and patients or grouped by controls and by disease. They were named controls, grouped MCADD and grouped MTPD. MCADD4 patient was excluded from the statistical calculations because it behaved differently from the other patients as seen when results were shown individually. The patients were diagnosed by metabolic centers by blood/urine biochemical markers and by gene sequencing at Research Unit for Molecular Medicine. Before use in the present project, they were taken from the diagnostic biobank and deidentified as required by the Danish Ethical Committee. Therefore only the information about the rare mutations necessary for classification as mild or severe is given.

1.2 Cell culturing

Cultured skin fibroblasts were cultivated in RPMI-1640 medium (Lonza) supplemented with 10% (v/v) fetal calf serum (Biological Industries), 1% penicillin/streptomycin (Leo Pharmaceutical), 2 mM of L-glutamine (Leo Pharmaceutical) in tissue flasks (TPP Techno Plastic Products AG) at 37^{o}C, 5% (v/v) CO_2 atmosphere until reach 80% confluence. For the experiments, the cells were seeded in 6

well plates. After 24 h, the seeded cells used for assays at standard growing conditions (11 mM glucose) had the medium replaced by fresh RPMI-1640. The cells were also submitted to a metabolic stress conditions with galactose or galactose plus palmitate instead of glucose. Briefly, the medium was replaced by RPMI medium supplemented with galactose (11 mM) without glucose, streptomycin/penicillin (1%), GlutaMax (1.5 mM) and dialyzed serum (10 %). This medium was referred in the text and figures as galactose medium (Gal). The galactose medium was also supplemented by palmitate (100 µM) and was referred as galactose plus palmitate medium (GalPalm). After 12 or 24 h of incubation in galactose or galactose plus palmitate media, the cells were incubated with MitoSOX and Sytox green as described below. Fibroblasts from each control or patient were cultured three times and individual results were expressed as mean and standard deviation from these three independent experiments.

1.3 Palmitate-BSA conjugation

We initially prepared 150 mM palmitic acid solution in ethanol. An aliquot (300 µL) of this preparation was then dissolved in 4% BSA solution (600 µM), prepared in RPMI, to achieve final molar ratio of 5:1 (FA: BSA). Conjugation was done at 37^{o}C for 1 h. BSA-fatty acid conjugates were further diluted in RPMI (without glucose) supplemented with galactose (11 mM), streptomycin/penicillin (1%), GlutaMax (1.5 mM) and dialyzed serum (10 %) until reach a final concentration of 100 µM of palmitate (GalPalm). To prepare the palmitate-free galactose medium (Gal), the same amount of 4 % BSA solution (not conjugated with palmitate) was added to the medium.

1.4 MytoSOX and Sytox measurements

The mitochondrial superoxide levels were measured by the positively charged fluoroprobe MitoSOX red (Invitrogen, USA) that is used for selective detection of superoxide inside mitochondria of living cells. MitoSOX accumulates inside the mitochondria as a membrane potential function and is oxidized by superoxide anion exhibiting red fluorescence. After cells incubation in RPMI or with RPMI containing galactose or galactose plus palmitate media during 12 or 24 h, the cells were washed three times and incubated with Hank's balanced salt solution (HBSS) and 5 μM MitoSOX for 20 minutes, at 37^{o}C. After MitoSOX incubation, the cells were washed three times with PBS and trypsinized. The cells were then re-suspended in Hoechst 33342 at final concentration of 10 μg/mL in HBSS and incubated for 15 minutes at 37^{o}C for nuclear staining. After incubation, 30 ng/mL Sytox green were added to stain dead cells. Sytox green is a green-fluorescent nuclear and chromosome counterstain that is impermeant to live cells, but easily permeant to cell with compromised membranes characteristic of dead cells. The cells suspension was then loaded into slides and the multicolor labeled fluorescence measurements were performed in Nucleo Counter NC -3000 (Chemometec, Denmark) in the excitation and emission wavelengths of Ex365-Em453, Ex475-Em560/35, Ex530-Em675/75. The NC-3000 software was used for image acquisition, analysis and quantification as well as data visualization. The MitoSOX measurements results are expressed as percentage of stressed cells defined as the MitoSOX intensity above the control levels. The Sytox measurements are expressed as fluorescence mean intensity. An illustrative image of fibroblasts stained with Hoechst, MytoSox and Sytox green is showed in Figure 1.

1.5 Statistical analysis

Results are presented as mean ± standard deviation. Data were analyzed using one-way analysis of variance (ANOVA) followed by the *post-hoc* Tukey's multiple comparison test when F was significant. Pearson coefficient of correlation was also used in some experiments. Differences between groups were rated significant at $P < 0.05$.All analyses were carried out using the GraphPad software.

2. Results

2.1 Mitochondrial superoxide is increased by metabolic stress conditions

First, we determined mitochondrial superoxide levels in skin fibroblasts from healthy individuals (controls) and patients affected by MCAD and MTP deficiencies under standard growing conditions (11 mM glucose). It is apparent from the Figure 2A (individual values) that two MCADD patients and all MTPD patients presented higher levels of superoxide (expressed as percentage of stressed cells) when compared to controls. We can also observe in Figure 2A that MTPD1 patient had the most stressed cells, as compared to controls, whereas fibroblasts from MCADD1 and MCADD4 patients were not stressed. Statistical analysis demonstrated that MTPD patients had significantly greater levels of mitochondrial superoxide production as compared to controls [$F_{(2,9)} = 9.86$; $P < 0.01$] and that MCADD patients had a nonsignificant increase of these levels (Figure 2B, grouped values). These results suggest that patients affected by these disorders, especially by MTPD, suffer from chronic oxidative stress under basal cultured conditions.

Next, we evaluated mitochondrial superoxide levels in cells under metabolic stress conditions incubated during 12 or 24 h in RPMI medium without glucose supplemented with galactose (Gal) or with galactose plus palmitate (GalPalm). We therefore investigated whether the presence of mutant

MCAD or MTP proteins represents a risk factor for superoxide production in fibroblasts from these patients under metabolic stress. It is of note that when cells are cultured in glucose containing medium ATP production is mainly supported by the glycolytic pathway even in an oxygen atmosphere, whereas when glucose is substituted by galactose the cells shift to mitochondrial oxidative phosphorylation as the main source of ATP. Furthermore, when the medium culture is supplemented with GalPalm, palmitate leads to the building up of intermediates usually found in biological fluids and tissues from MCAD or MTP deficient patients, what represents an additional stressor factor for the cells.

We observed in Figure 3A (individual values) and 3B (grouped values) significantly increased superoxide levels in fibroblasts from MCADD and MTPD patients using either Gal or GalPalm medium after 12 h incubation, as compared to the controls [Gal: $F_{(2,8)} = 14.74$; $P < 0.01$; GalPalm: $F_{(2,9)} = 17.71$; $P < 0.01$]. Moreover, there were no differences between superoxide levels in stressed fibroblasts from MCADD and MTPD patients under both stressed conditions (Gal or GalPalm) (Figure 2B).

Furthermore, when cells where kept under metabolic stress in Gal medium during 24 h, similar results were achieved (Figure 3C and 3D). It is also observed that control fibroblasts supplemented by galactose plus palmitate nearly doubled the levels of superoxide, as compared to the same cells with solely galactose in the medium. It is also seen in Figure 3A and 3C that MCADD4 superoxide values did not differ from controls under both incubation times and metabolic stress conditions.

2.2 MTPD cells are more prone to death

In the next set of experiments, we analyzed cell death in cultured fibroblasts from MCADD and MTPD patients under standard and metabolic stress conditions by using Sytox green. Figure 4A and 4B

shows that fibroblasts from MTPD patients (particularly from MTPD1 and 4) under standard conditions, are more prone to death, as compared to controls and to MCADD patients [$F_{(2,9)} = 11.98$; $P < 0.01$]. It is also of note that cell death was similar or even lower in MCADD4 patient, relatively to controls (Figure 4A).

When cells were stressed by supplementing the cultures with Gal or Gal plus Palm for 12 h or 24 h, similar results were found, i.e., MTPD stressed fibroblasts presented a higher cell death index in comparison to controls and MCADD patients [12 h: Gal: $F_{(2,9)} = 9.91$; $P < 0.01$; GalPalm: $F_{(2,9)} = 12.72$; $P < 0.01$] [24 h: Gal: $F_{(2,9)} = 10.71$; $P < 0.01$; GalPalm: $F_{(2,9)} = 12.18$; $P < 0.01$] (Figure 5). There were also no differences in cell death between the different stress conditions (Gal and GalPalm) or time of incubation. It was also observed that cell death under basal (Figure 4) and stressed conditions (Figure 5) did not differ in a significant way, implying that Gal or GalPalm supplementation in the cultured medium did not increase cell mortality.

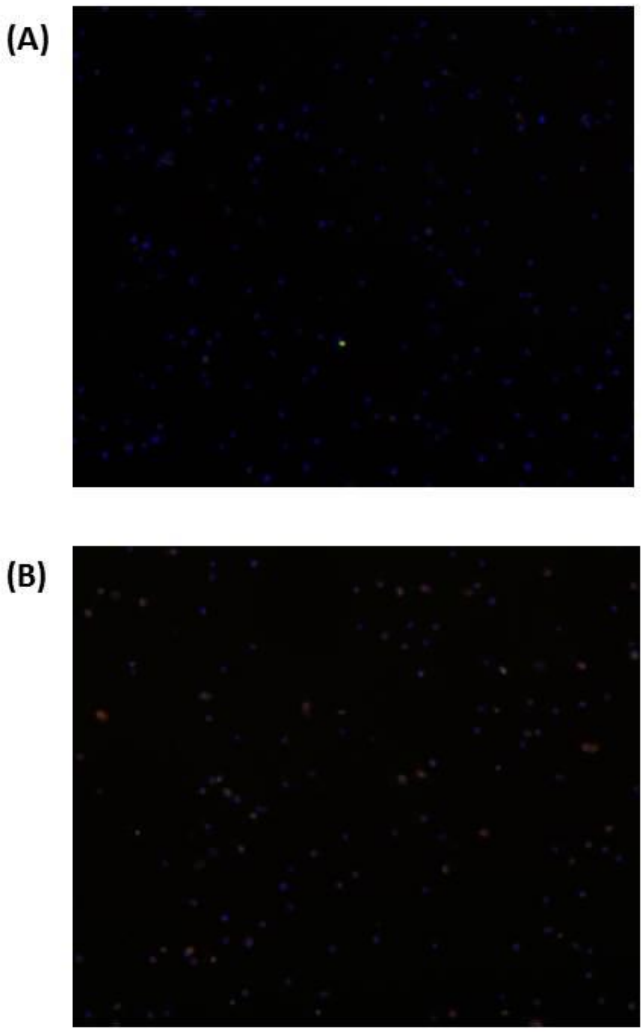

Figure 1. Illustrative image of fibroblasts stained with MytoSox and Sytox green in Nucleo Counter NC - 3000. Fibroblasts were stained with Hoechst (blue fluorescence), MytoSox (orange fluorescence) and Sytox green (green fluorescence). (A) Fibroblasts from Control2 after 12 h incubation in galactose media; (B) Fibroblasts from patient MTPD2 after 12 h incubation in galactose media.

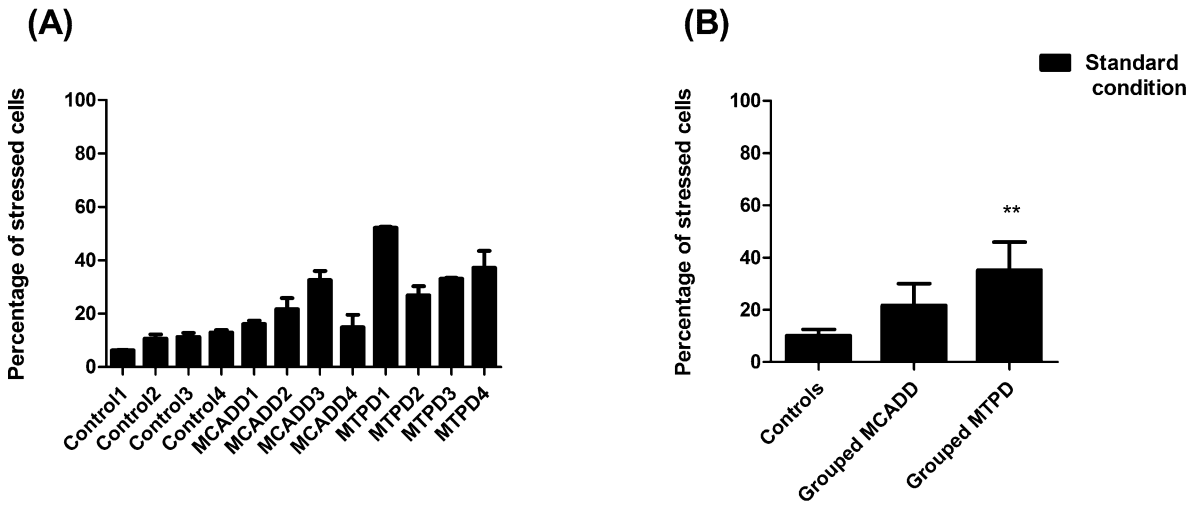

Figure 2. Comparison between superoxide generation in cultured skin fibroblasts from controls (control 1 - 4) and patients with medium-chain acyl-CoA dehydrogenase (MCADD 1 - 4) and mitochondrial trifunctional protein deficiencies (MTPD 1 - 4) (A) individual values; (B) grouped values: controls 1 - 4, MCADD 1 - 3 and MTPD 1 - 4 patients. Cells were cultivated in RPMI medium (standard conditions – 11 mM glucose). Superoxide increase was estimated by the MitoSOX probe. Results are expressed as percentage of stressed cells defined as MitoSOX intensity above the control average level. Values are mean ± standard deviation for three independent experiments (A) or three to four experiments (B). MCADD4 patient was not included in the calculations for grouped values. $^{**}P < 0.01$ compared to controls (Tukey's multiple comparison test).

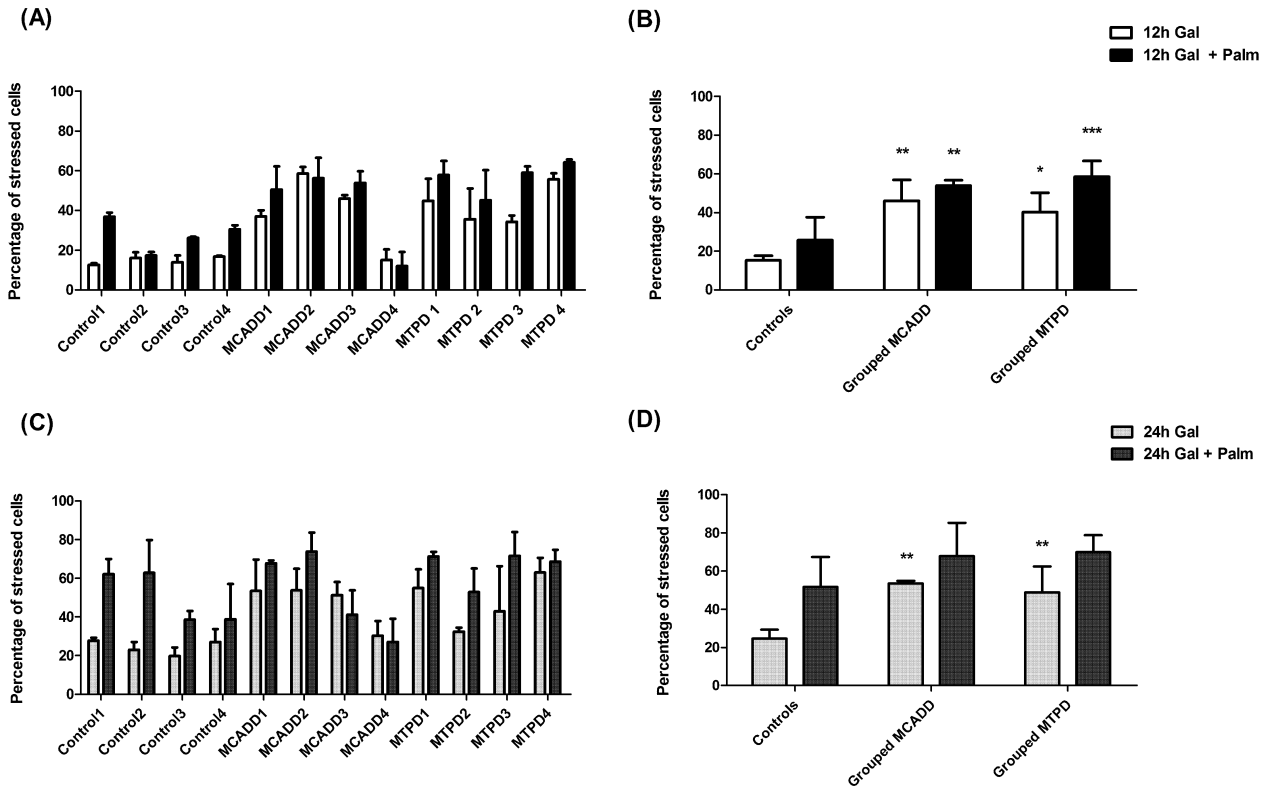

Figure 3. Comparison between superoxide generation in cultured skin fibroblasts from controls (control 1 - 4) and patients with medium-chain acyl-CoA dehydrogenase (MCADD 1 - 4) and mitochondrial trifunctional protein deficiencies (MTPD 1 - 4) (A,C) individual values; (B,D) grouped values: controls 1 - 4, MCADD 1 - 3 and MTPD 1 - 4 patients. Cells were cultivated in RPMI medium without glucose supplemented with 11 mM galactose (Gal) or 11 mM galactose plus 100 μM palmitate (Gal + Palm), during 12 h (A,B) or 24 h (C,D). Superoxide increase was estimated by MitoSOX probe. Results are expressed as stressed cells defined as the MitoSOX intensity above the controls level. Values are mean ± standard deviation for three independent experiments (A) or three to four experiments (B). MCADD4 patient was not included in the calculations for grouped values. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to controls (Tukey's multiple comparison test).

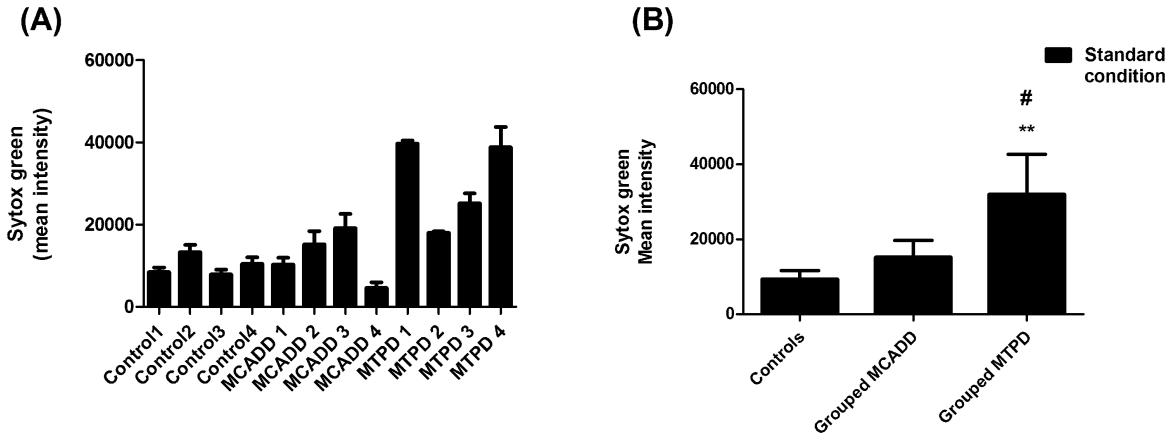

Figure 4. Comparison between sytox green intensity in cultured skin fibroblasts from controls (control 1 - 4) and patients with medium-chain acyl-CoA dehydrogenase (MCADD 1 - 4) and mitochondrial trifunctional protein deficiencies (MTPD 1 - 4) (A) individual values; (B) grouped values: controls 1 - 4, MCADD 1 - 3 and MTPD 1 - 4 patients. Cells were cultivated in RPMI (standard growing conditions – 11 mM of glucose). Results are expressed as sytox green mean intensity. Values are mean ± standard deviation for three independent experiments (A) or three to four experiments (B). MCADD4 patient was not included in the calculations for grouped values. $^{**}P < 0.01$ compared to controls, $^{\#}P < 0.05$ compared to grouped MCADD (Tukey's multiple comparison test).

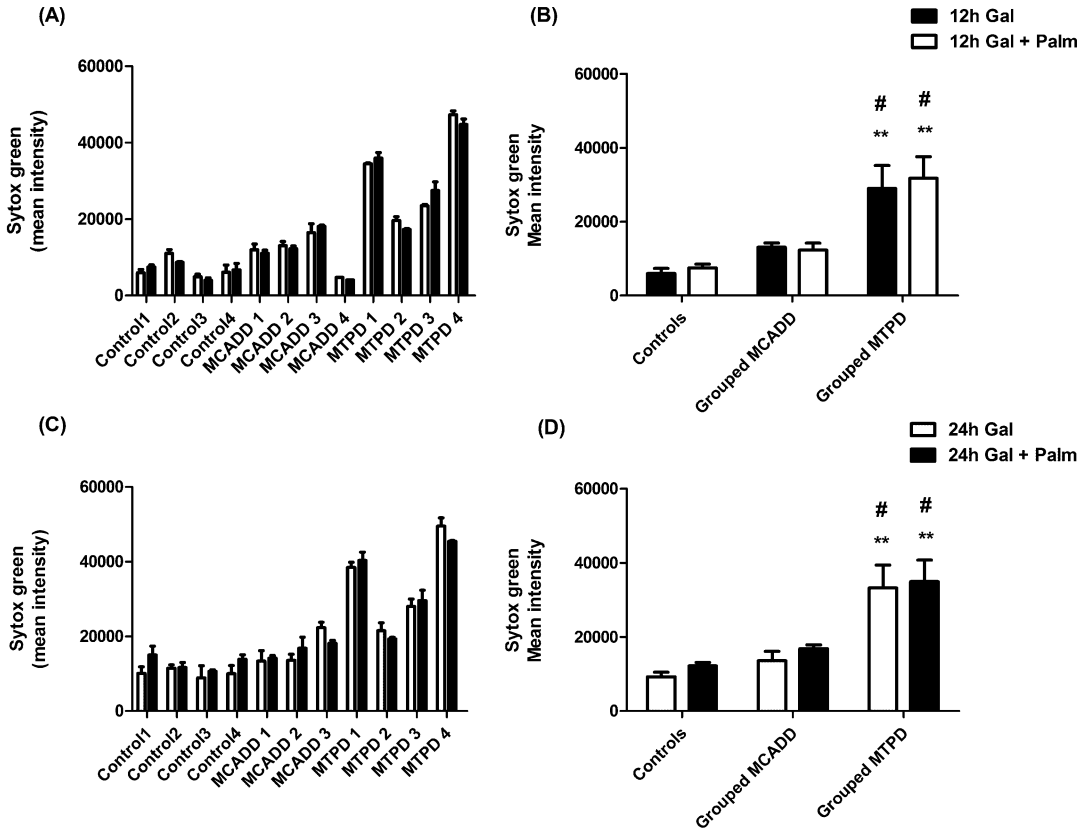

Figure 5. Comparison between sytox green intensity in cultured skin fibroblasts from controls (control 1 - 4) and patients with medium-chain acyl-CoA dehydrogenase (MCADD 1 - 4) and mitochondrial trifunctional protein deficiencies (MTPD 1 - 4) (A,C) individual values; (B,D) grouped values: controls 1 - 4, MCADD 1 - 3 and MTPD 1 - 4 patients. Cells were cultivated in RPMI without glucose supplemented with 11 mM galactose (Gal) or 11 mM galactose plus 100 μM palmitate (Gal + Palm), during 12 h (A,B) and 24 h (C,D). Results are expressed as sytox green mean intensity. Values are mean ± standard deviation for three independent experiments (A) or three to four experiments (B). MCADD4 patient was not included in the calculations for grouped values. $^{**}P < 0.01$ compared to controls, $^{\#}P < 0.05$ compared to grouped MCADD (Tukey's multiple comparison test).

CHAPTER III

Long-chain 3-hydroxy fatty acids accumulating in long-chain 3-hydroxyacyl-CoA dehydrogenase and mitochondrial trifunctional protein deficiencies uncouple oxidative phosphorylation in heart mitochondria

Anelise M Tonin, Alexandre U. Amaral, Estela N. Busanello, Mateus Grings. Roger F. Castilho, Moacir Wajner

Scientific article published in

Journal of Bioenergetics and Biomembranes 45(1-2): 45-57, 2013

J Bioenerg Biomembr (2013) 45:47–57
DOI 10.1007/s10863-012-9481-9

Long-chain 3-hydroxy fatty acids accumulating in long-chain 3-hydroxyacyl-CoA dehydrogenase and mitochondrial trifunctional protein deficiencies uncouple oxidative phosphorylation in heart mitochondria

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Received: 25 July 2012 / Accepted: 2 October 2012 / Published online: 13 October 2012

Abstract Cardiomyopathy is a common clinical feature of some inherited disorders of mitochondrial fatty acid β-oxidation including mitochondrial trifunctional protein (MTP) and isolated long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiencies. Since individuals affected by these disorders present tissue accumulation of various fatty acids, including long-chain 3-hydroxy fatty acids, in the present study we investigated the effect of 3-hydroxydecanoic (3 HDCA), 3-hydroxydodecanoic (3 HDDA), 3-hydroxytetradecanoic (3 HTA) and 3-hydroxypalmitic (3 HPA) acids on mitochondrial oxidative metabolism, estimated by oximetry, NAD(P)H content, hydrogen peroxide production, membrane potential (ΔΨ) and swelling in rat heart mitochondrial preparations. We observed that 3 HTA and 3 HPA increased resting respiration and diminished the respiratory control and ADP/O ratios using glutamate/malate or succinate as substrates. Furthermore, 3 HDDA, 3 HTA and 3 HPA decreased ΔΨ, the matrix NAD(P)H pool and hydrogen peroxide production. These data indicate that these fatty acids behave as uncouplers of oxidative phosphorylation. We also verified that 3 HTA-induced uncoupling-effect was not mediated by the adenine nucleotide translocator and that this fatty acid induced the mitochondrial permeability transition pore opening in calcium-loaded organelles since cyclosporin A prevented the reduction of mitochondrial ΔΨ and swelling provoked by 3 HTA. The present data indicate that major 3-hydroxylated fatty acids accumulating in MTP and LCHAD deficiencies behave as strong uncouplers of oxidative phosphorylation potentially impairing heart energy homeostasis.

Keywords Long-chain 3-hydroxy fatty acids · Mitochondria · Heart bioenergetics · Oxidative phosphorylation · Permeability transition

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Introduction

The mitochondrial trifunctional protein (MTP) is an enzymatic complex associated with the inner mitochondrial membrane and participates in the β-oxidation of long-chain fatty acids. MTP comprises the activities of 3-hydroxyacyl-CoA dehydrogenase (LCHAD), 2-enoyl-CoA hydratase and 3-oxoacyl-CoA thiolase (LKAT). Molecular defects in MTP-enconding genes lead to deficiencies of isolated enzymatic activities or a deficiency affecting the activities of all three enzymatic components characterizing the

MTP deficiency (Rinaldo et al. 2002). These diseases are biochemically characterized by tissue accumulation and high excretion in urine of long-chain 3-hydroxyacylcarnitines and long-chain 3-hydroxy fatty acids (LC3HFA) including 3-hydroxydecanoic (3 HDCA), 3-hydroxydodecanoic (3 HDDA), 3-hydroxytetradecanoic (3 HTA) and 3-hydroxypalmitic (3 HPA) acids (Jones et al. 2001; Hintz et al. 2002; Sander et al. 2005).

Patients have been described with either MTP deficiency or more commonly with an isolated LCHAD deficiency. Only few cases of LKAT deficiency have been related (Sander et al. 2005; Das et al. 2006). Impaired fatty acid oxidation (FAO) activity causes a shortage of energy, particularly in circumstances of high energy demand such as postnatal period, prolonged fasting, exercise, infection and inflammatory process. Generally, the symptoms presented by patients are severe and have been associated with energetic deficiency particularly in FAO-dependent tissues, such as the heart, liver and muscle, and also with potential toxic properties of the intermediary metabolites of FAO present at elevated intracellular concentrations (Ventura et al. 1998; Jones et al. 2001).

Cardiac involvement with cardiomyopathy and arrhythmias are frequent findings in MTP and LCHAD deficiencies and may appear suddenly as ventricular arrhythmias during an acute episode or a more chronic progression with cardiomyopathy may develop (Huss and Kelly 2005; Ventura et al. 1995, 1996). They can be also associated with Reye-like syndrome, lactic acidemia and neonatal death (Moczulski et al. 2009).

The pathogenic mechanisms involved in heart dysfunction in these disorders are still not fully established. However, long-chain acylcarnitines were shown to cause myocardial injury and rhythm disturbances during myocardial ischemia and infarction (Corr et al. 1989). Moreover, previous studies have shown deleterious effects of long-chain fatty acids and their derivatives to energy metabolism. In this regard, ATP synthesis was demonstrated to be inhibited by palmitoyl-CoA and its 3-hydroxy and 3-keto derivatives in human fibroblasts (Ventura et al. 1995, 1996). Long chain acyl-CoA esters have also been shown to inhibit oxidative phosphorylation, the mitochondrial ATP/ADP translocator and the dicarboxylate carrier, as well as respiratory chain enzyme activities in isolated rat liver mitochondria (Ventura et al. 2005, 2007). Furthermore, our group has recently reported that LC3HFA induces oxidative stress and disrupt mitochondrial homeostasis in rat brain (Tonin et al. 2010a, b).

However, to the best of our knowledge, nothing has been reported on the role of these fatty acids on heart function and biochemistry. Therefore, in the present work we evaluated the effects of the LC3HFA, 3 HDCA, 3 HDDA, 3 HTA and 3 HPA on important parameters of mitochondrial bioenergetics in the heart, including ADP-stimulated (state 3) and resting (state 4) mitochondrial respiration, the respiratory control ratio (RCR) and ADP/O ratio, as well as NAD(P)H content, hydrogen peroxide production, membrane potential and swelling in heart mitochondrial preparations from young rats.

Material and methods

Reagents

All chemicals were purchased from Sigma (St. Louis, MO, USA). 3 HDCA, 3 HDDA, 3 HTA and 3 HPA were dissolved previously in methanol and, on the day of the experiments, were diluted with specific buffers and the pH was adjusted to 7.4. The final concentrations of these fatty acids in the medium ranged from 10 μM to 100 μM. The final concentration of methanol in the incubation medium in the absence (controls) and presence of these fatty acids (experimental groups) was always 1 %. This concentration was shown not to alter the examined parameters.

Animals

Thirty-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, were used. Rats were kept with dams until weaning at 21 days of age. The animals were maintained on a 12:12 h light/dark cycle (lights on 07.00–19.00 h) at an air conditioned constant temperature (22±1 °C) colony room, with free access to water and 20 % (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil and followed the Principles of Laboratory Animal Care (NIH publication 85–23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

Preparation of heart mitochondrial fractions

Heart mitochondria were isolated according to the method of Ferranti et al. (Ferranti et al. 2003) with slight modifications. Animals were euthanized by decapitation, hearts were removed, and immediately washed in ice cold isolation buffer containing 225 mM mannitol, 75 mM sucrose,1 mM EGTA, and 10 mM HEPES, pH 7.2. The tissue was finely minced and incubated in the presence of protease type I for 10 min. Excess of protease was removed by washing the heart fragments with the isolation buffer

containing 1 mg/mL bovine serum albumin (BSA). The tissue was then homogenized (1:10 w/v) manually and centrifuged at 600 × g for 5 min. The supernatant was then centrifuged at 9,000 × *g* for 8 min to obtain the mitochondrial pellet. The pellet was washed twice to eliminate contaminating blood and resuspended in 400 μL of isolation buffer devoid of EGTA.

We always carried out parallel experiments with various blanks (controls) in the presence or absence of 3 HDCA, 3 HDDA, 3 HTA or 3 HPA and also with or without mitochondrial preparations in the reaction medium to detect any interference (artifacts) of these fatty acids on the techniques utilized to measure the mitochondrial parameters.

Respiratory parameters determined through mitochondrial oxygen consumption

The rate of oxygen consumption was measured polarographically using a Clark-type electrode in a thermostatically controlled (37 °C) and magnetically stirred incubation chamber. 3 HDCA, 3 HDDA, 3 HTA or 3 HPA (10–100 μM) were added to the reaction medium at the beginning of the assay. The assay was performed with purified mitochondrial preparations (0.2 mg protein^{-1}. mL^{-1}) using 2.5 mM glutamate plus 2.5 mM malate or 5 mM succinate plus 4 μM rotenone as substrates and incubated for 60 s in a buffer containing 0.3 M sucrose, 5 mM MOPS, 5 mM potassium phosphate, 1 mM EGTA and 0.1 % BSA, pH 7.4. State 3 respiration was measured after addition of 1 mM ADP to the incubation medium. To measure resting (state 4) respiration, 1 μg. mL^{-1} oligomycin A was added to the incubation medium. The respiratory control ratio (RCR: state 3/state 4) was then calculated. States 3 and 4 were expressed as nmol O_2 consumed. min^{-1}. mg protein^{-1}. The ADP/O ratio was estimated according to Estabrook (Estabrook 1967), using 100 μM ADP in the incubation medium. Only mitochondrial preparations with RCR greater than 4 were used in the experiments.

Determination of NAD(P)H fluorescence

Matrix mitochondrial NAD(P)H autofluorescence was measured at 37 °C using 366 nm excitation and 450 nm emission wavelengths on a Hitachi F-4500 spectrofluorometer. State 4 respiring mitochondria (0.2 mg. protein^{-1}. mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate were incubated in a standard reaction medium containing 125 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.01 % BSA, 5 mM HEPES, 2 mM KH_2PO_4, pH 7.2. 3 HDCA, 3 HDDA, 3 HTA and 3 HPA (10–100 μM) were added to the reaction medium 100 s after the beginning of the assay and rotenone (4 μM) was added at the end of the measurements. Data were expressed as fluorescence arbitrary units (FAU).

Mitochondrial hydrogen peroxide (H_2O_2) release

Mitochondrial preparations (0.2 mg protein^{-1}. mL^{-1}) supported by 5 mM succinate were incubated in standard reaction medium in the presence of 10 μM Amplex red and 1 U/mL horseradish peroxidase. The fluorescence was monitored over time on a Hitachi F-4500 spectrofluorometer operated at excitation and emission wavelengths of 563 and 587 nm, respectively, and slit widths of 5 nm. 3 HDCA, 3 HDDA, 3 HTA and 3HPA (10–100 μM) were added to the reaction medium 100 s after the beginning of the assay. Antimycin A (AA) (0.1 μg. mL^{-1}) was added at the end of the measurements. Data were expressed as FAU.

Determination of the mitochondrial transmembrane electrical potential

Mitochondrial inner membrane potential ($\Delta\Psi$) was estimated according to Akerman and Wikström (Akerman and Wikström 1976) and Kowaltowski et al. (Kowaltowski et al. 2002) using state 4 respiring mitochondria (0.3 mg. protein^{-1}. mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate in a standard reaction medium. 3 HDCA, 3 HDDA, 3 HTA and 3HPA (10–100 μM) were added to the reaction medium 100 s after the beginning of the assay. In some experiments atractyloside (ATC, 30 μM) was used in the assays. Other assays were carried out with Ca^{2+} (20 μM) and/or cyclosporin A (CsA, 1 μM) supplemented to the reaction medium with a small concentration of EGTA (30 μM) enough to scavenge endogenous calcium. CCCP (1 μM) was always added at the end of the measurements. The fluorescence of 5 μM cationic dye safranin O at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 10 nm, was followed for 5 min on a Hitachi F-4500 spectrofluorometer. Data were expressed as FAU.

Determination of mitochondrial swelling

Mitochondrial swelling was followed by measuring light scattering changes on a temperature-controlled Hitachi F-4500 spectrofluorometer with magnetic stirring operating at excitation and emission of 540 nm using state 4 respiring mitochondria (0.3 mg. protein^{-1}. mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate in a standard reaction medium. The assays were carried out with Ca^{2+} (200 μM) and/or CsA (1 μM) in a reaction medium with a small concentration of EGTA (30 μM) enough to scavenge endogenous calcium. Alamethicin (Alm, 40 μg/mL) was always added at the end of the measurements. Data were expressed as FAU.

Protein determination

Protein was measured by the method of Bradford et al. (Bradford 1976) using BSA as standard.

Statistical analysis

Results are presented as mean±SD. Assays were performed in duplicate and the mean was used for statistical analysis. Mitochondria isolated from heart of four animals were utilized for each technique. Data were analyzed by one-way ANOVA, followed by the post hoc Duncan multiple range test when F was significant. Only significant F values are shown in the text. Differences between groups were rated significant at $P<0.05$. All analyses were carried out using the Statistical Package for the Social Sciences (SPSS) software.

Results

LC3HFA increased state 4 mitochondrial respiration and reduced RCR and ADP/O ratio

We first verified that rat heart mitochondria incubated under our conditions were well functioning, as indicated by the higher respiratory rates observed in the presence of ADP (state 3) relatively to those obtained after the addition of oligomycin (state 4 or resting respiration) (Figs. 1, 2 and 3).

We first verified that state 4 respiration was increased in a dose dependent manner by 3 HTA (up to 194 %) and 3 HPA (up to 96 %), but not by 3 HDCA and 3 HDDA (Fig. 1), regardless of the substrate used [glutamate/malate (3HTA: $F_{(4,10)}=68.45$, $P<0.001$; $\beta=0.850$, $P<0.001$; 3HPA: $F_{(4,20)}=32.25$, $P<0.001$; $\beta=0.845$, $P<0.001$); succinate (3HTA: $F_{(4,10)}=6.45$, $P<0.01$; $\beta=0.810$, $P<0.001$; 3HPA: $F_{(4,10)}=57.97$, $P<0.001$; $\beta=0.903$, $P<0.001$)] as observed in Fig. 1c and d. In contrast, state 3 respiration was not changed by any LC3HFA (Fig. 2). The mitochondrial RCR was also decreased by 3 HTA (up to 72 %) and 3 HPA (up to 51 %) with glutamate/malate [3HTA: $F_{(4,10)}=39.88$; $P<0.001$; 3HPA: $F_{(4,20)}=6.10$; $P<0.01$] and succinate [3HTA: $F_{(4,10)}=8.84$; $P<0.01$; 3HPA: $F_{(4,10)}=49.25$], as seen in Fig. 3c and d.

We next found that 3 HTA significantly reduced the ADP/O ratio (up to 39 %) at the concentrations of 50 μM and 100 μM with glutamate/malate [3HTA: $F_{(2,6)}=66.90$; $P<0.001$] and succinate [3HTA: $F_{(2,9)}=6.22$; $P<0.01$] (Fig. 4), suggesting a decrease in the oxidative phosphorylation efficiency (Du et al. 1998). These data indicate that uncoupling of mitochondria is the explanation that better fits the alterations observed in the respiratory parameters.

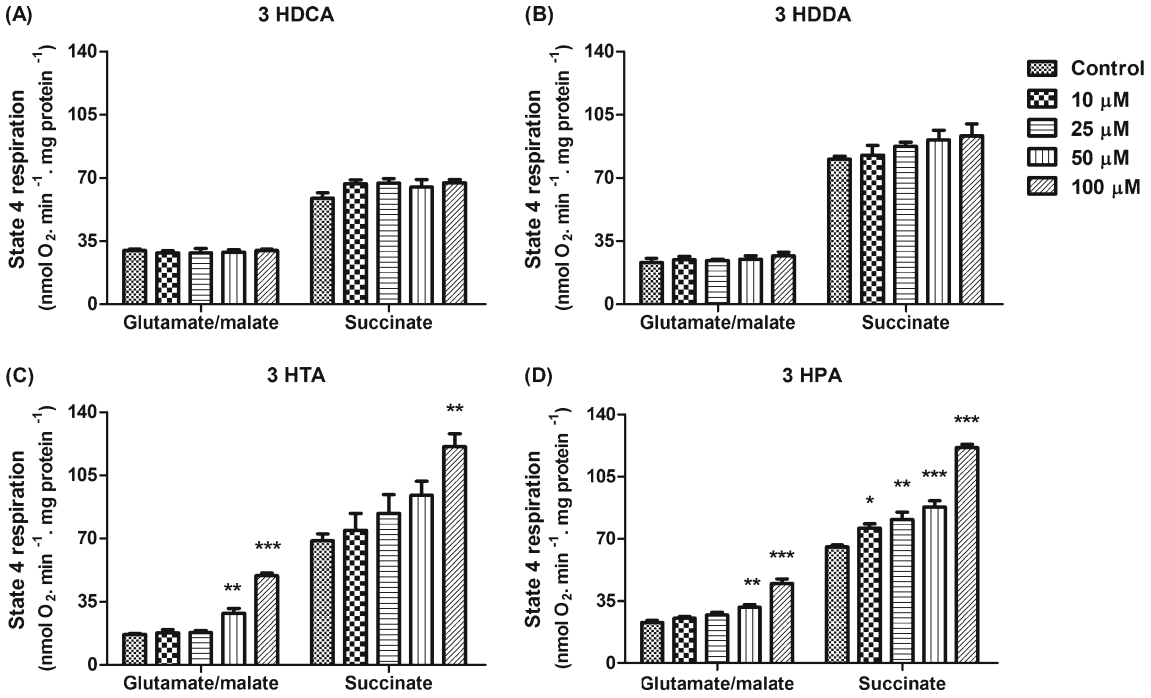

Fig. 1 Effect of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on state 4 respiration using glutamate/malate or succinate plus rotenone (4 μM) as substrates. Heart mitochondrial preparations (0.2 mg protein. mL^{-1}) and various concentrations of 3 HDCA, 3 HDDA, 3 HTA or 3 HPA were added to the incubation medium in the beginning of the assays in the presence of oligomycin (1 μg. mL^{-1}). The control group did not contain these fatty acids in the medium. Values are means±standard deviation for four independent experiments and are expressed as nmol O_2. min^{-1}. mg of $protein^{-1}$. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared to controls (Duncan multiple range test)

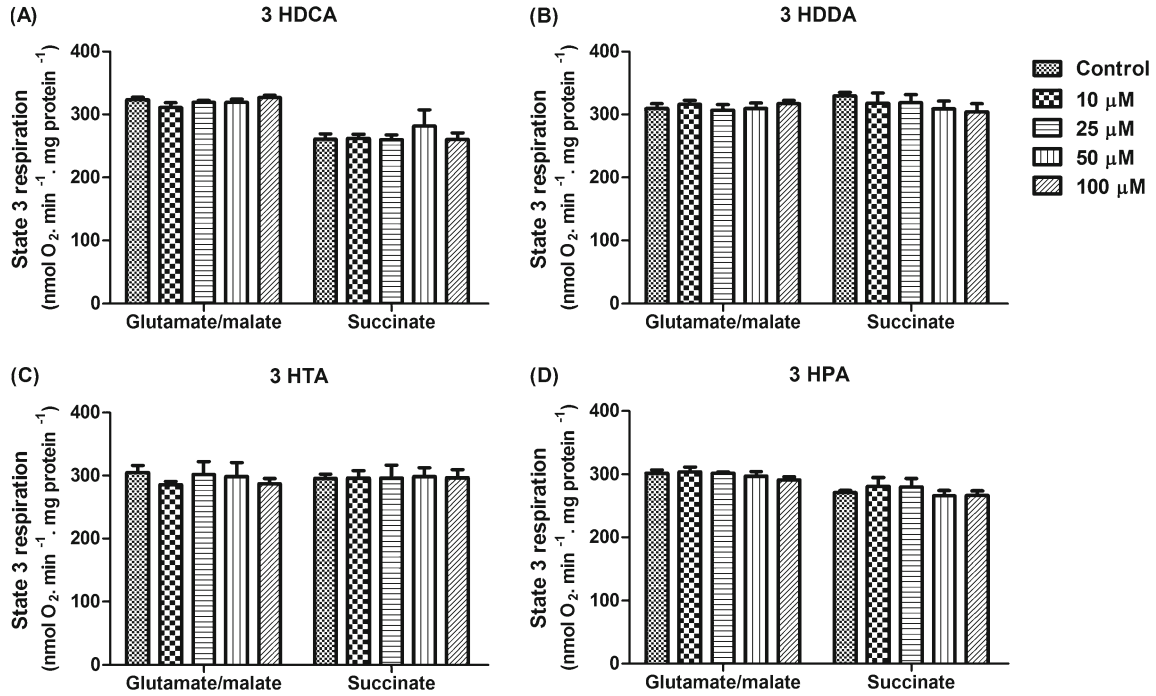

Fig. 2 Effects of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on state 3 respiration using glutamate/malate or succinate plus rotenone (4 μM) as substrates. Heart mitochondrial preparations (0.2 mg protein. mL^{-1}) and different concentration of 3 HDCA, 3 HDDA, 3 HTA or 3 HPA were added in the incubation medium in the beginning of the assay in the presence of 1 mM ADP. The control group did not contain these fatty acids in the medium. Values are means±standard deviation for four independent experiments and are expressed as nmol O_2. min^{-1}. mg of $protein^{-1}$

Mitochondrial matrix NAD(P)H pool was reduced by LC3HFA

We then assayed the effects of LC3HFA on mitochondrial NAD(P)H content, since uncouplers of oxidative phosphorylation reduce the matrix reduced equivalent pool. The mitochondria incubated with 3 HDDA, 3 HTA or 3 HPA resulted in a decreased mitochondrial NAD(P)H pool (Fig. 5).

LC3HFA diminished hydrogen peroxide production

Next we evaluated whether the LC3HFA were able to alter the hydrogen peroxide (H_2O_2) production in the presence of succinate as substrate. We found that 3 HDDA, 3 HTA and 3 HPA significantly reduced H_2O_2 production (Fig. 6), which is also caused by uncouplers. The inhibitory effect of LC3HFA on mitochondrial H_2O_2 production was abolished by the addition of the respiratory chain complex III inhibitor antimycin A.

LC3HFA decreased the inner mitochondrial membrane potential

The inner mitochondrial membrane potential (ΔΨ) was also determined in the presence of LC3HFA in state 4 respiration using glutamate/malate as substrates. 3 HDDA, 3 HTA and 3 HPA markedly diminished the ΔΨ in a dose–response manner, as depicted in Fig. 7. Furthermore, 3 HDCA caused no effects, whereas 3 HDDA-induced reduction of ΔΨ was less pronounced. It can be also seen in the figure that further addition of the proton ionophore CCCP was not able to change the fluorescence levels (ΔΨ) caused by 50 or 100 μM of 3 HTA and 3 HPA, indicating a potent uncoupling effect of these fatty acids.

We also supplemented the medium with 30 μM of atractyloside, an inhibitor of the adenine nucleotide translocator (ANT), in order to elucidate the mechanism by which 3 HTA uncouples mitochondria. We observed that atractyloside did not prevent the ΔΨ reduction induced by 3 HTA, ruling out a selective mitochondrial depolarization via ANT (Fig. 8). Interestingly, other fatty acids have been shown to uncouple oxidative phosphorylation via ANT (Schonfeld et al. 1989; Mokhova and Khailova 2005), implying that the LC3HFA use a distinct mechanism to uncouple mitochondria.

3 HTA provoked mitochondrial permeability transition in the presence of Ca^{2+}

We also observed that 3 HTA at a low dose (10 μM) provoked mitochondrial depolarization, which was markedly increased by the addition of 20 μM Ca^{2+} in the incubation medium (Fig. 9). It can be also seen in the figure that Ca^{2+}

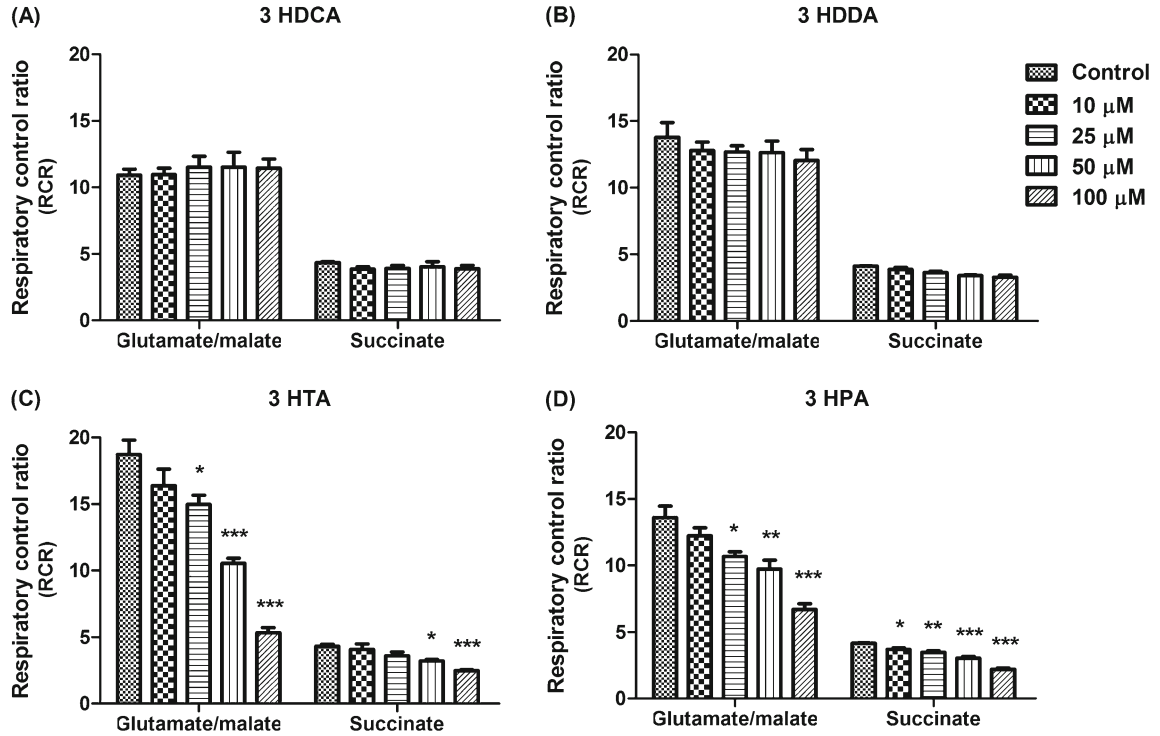

Fig. 3 Effects of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on respiratory control ratio (RCR) using glutamate/malate or succinate plus rotenone (4 μM) as substrates. Heart mitochondrial preparations (0.2 mg protein. mL^{-1}) and different concentration of 3 HDCA, 3 HDDA, 3 HTA or 3 HPA were added in the incubation medium. The control group did not contain these fatty acids in the medium. RCR was calculated. Values are means±standard deviation for four independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared to controls (Duncan multiple range test)

only slightly altered ΔΨ in the absence of 3HTA. Furthermore, the synergistic effect of 3HTA and Ca^{2+} inducing ΔΨ reduction was prevented by CsA, indicating the participation of mitochondrial permeability transition in 3 HTA effects on ΔΨ in heart mitochondrial preparations supplemented by Ca^{2+}.

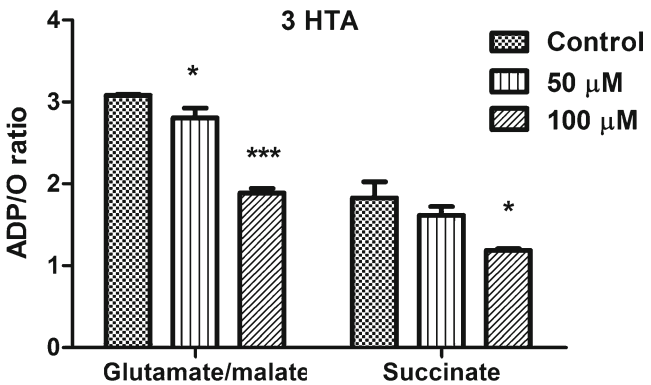

Fig. 4 Effect of 3-hydroxytetradecanoic acid (3 HTA) on ADP/O ratio using glutamate/malate or succinate plus rotenone (4 μM) as substrates. Heart mitochondrial preparations (0.2 mg protein. mL^{-1}) and 3 HTA (50 and 100 μM) were added to the incubation medium. The control group did not contain these fatty acids in the medium. ADP/O ratio was calculated. Values are means±standard deviation for four independent experiments. *$P<0.05$, ***$P<0.001$, compared to controls (Duncan multiple range test)

In an attempt to confirm that the reduction of ΔΨ caused by 3 HTA was indeed related to non-selective inner membrane permeabilization due to mitochondrial permeability transition, we measured mitochondrial swelling in the presence of 3 HTA, Ca^{2+} and/or CsA (Fig. 10). Alamethicin, which forms large pores in the inner mitochondrial membrane, was added at the end of each trace to cause maximal swelling. We found that 3 HTA caused a significant mitochondrial swelling after the addition of Ca^{2+} to the medium that was prevented by CsA, implying the participation of mitochondrial permeability transition in this effect.

Discussion

The exact pathomechanisms of tissue damage in MTP and LCHAD deficiencies are so far poorly established. However, the observations of lactic acidemia, inhibition of various complexes of the respiratory chain and mitochondrial morphological abnormalities in patients affected by these diseases point to a compromised mitochondrial function (Tyni et al. 1996; Ventura et al. 1998; Das et al. 2000;

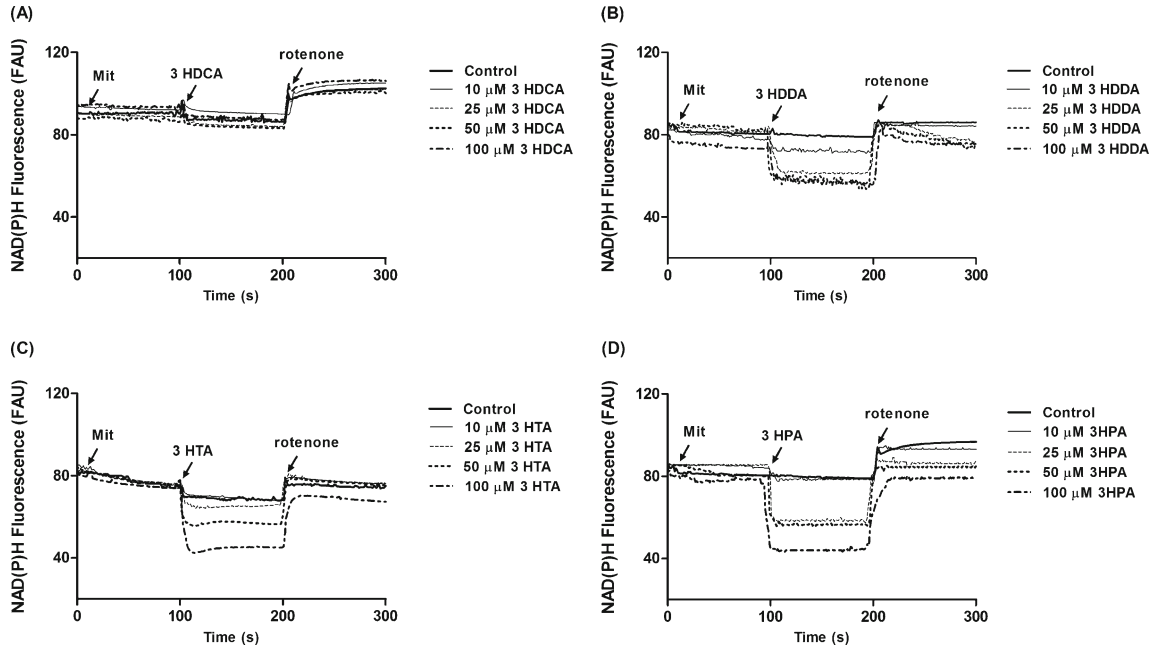

Fig. 5 Effects of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on NAD(P)H content using glutamate/malate as substrates. Heart mitochondrial preparations (Mit.; 0.2 mg protein. mL^{-1}) and various concentrations of 3 HDCA, 3 HDDA, 3 HTA or 3 HPA were added in the incubation medium in 100 s, as indicated by the arrows. The control group did not contain these fatty acids in the medium. Rotenone (4 μM) was added at the end of the assay, as indicated. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU)

Spiekerkoetter et al. 2008). Furthermore, it has been postulated that accumulation of long-chain acyl-CoA esters and β-oxidation intermediates, including the LC3HFA, may represent a major contributing factor for the clinical and biochemical phenotypes in long-chain fatty acid oxidation deficient patients, by causing impairment of energy

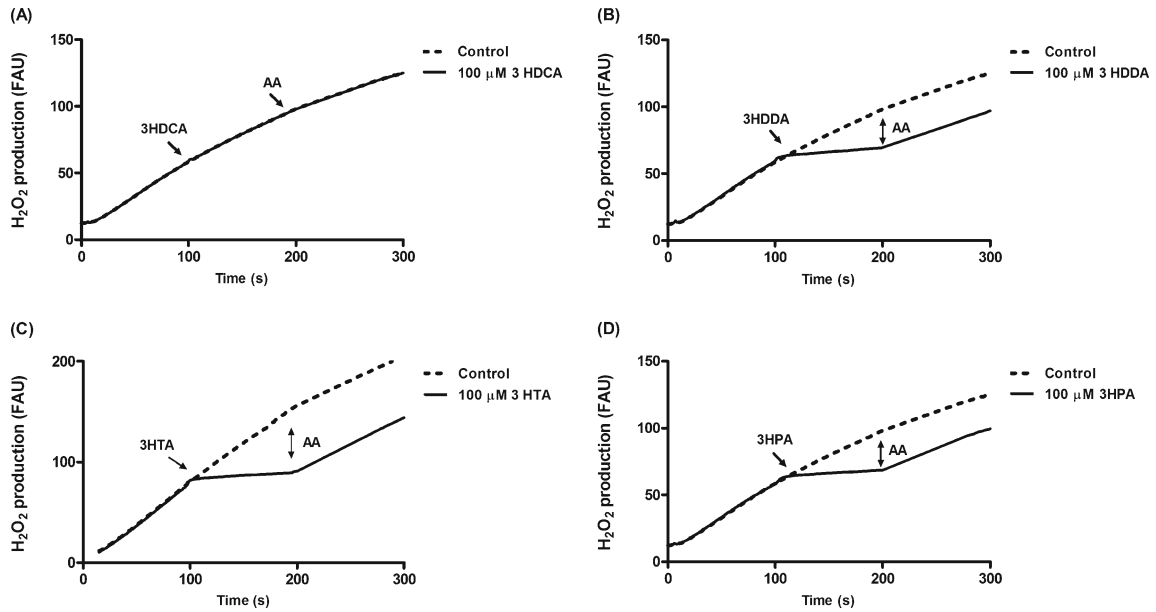

Fig. 6 Effects of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on hydrogen peroxide production using 5 mM succinate as substrate. Heart mitochondrial preparations (0.2 mg protein. mL^{-1}) were added to the incubation medium in the beginning of the assay. 3 HDCA, 3HDDA, 3 HTA or 3 HPA (100 μM) were added as indicated by the arrows. The control group did not contain these fatty acids in the medium. Antimycin A (AA) (0.1 μg. mL^{-1}) was added at the end of assays, as indicated. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU)

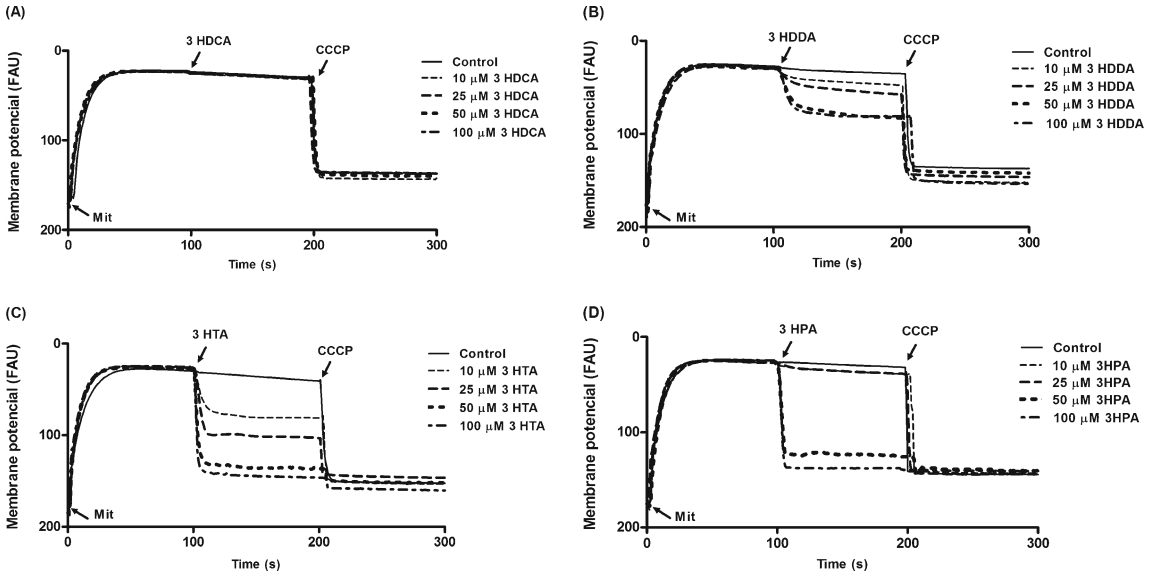

Fig. 7 Effects of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on mitochondrial membrane potential (ΔΨ) using glutamate/malate as substrates. Heart mitochondrial preparations (Mit., 0.3 mg protein. mL^{-1}) and various concentrations of 3 HDCA, 3HDDA, 3 HTA or 3 HPA were added to the incubation medium, as indicated by the arrows, in the presence of 5 μM safranine O and 1 μg. mL^{-1} oligomicyn. The control group did not contain these fatty acids in the medium. CCCP (1 μM) was added at the end of the assays, as indicated. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU)

homeostasis (Ventura et al. 2007). Our recent findings showing that LC3HFA induce oxidative stress and disrupt mitochondrial energy homeostasis in rat brain support this hypothesis (Tonin et al. 2010a, b).

Although the pathogenetic mechanisms involved in the development of cardiomyopathy in patients with MTP and LCHAD deficiencies are poorly understood, possible causes include inadequate energy supply in the heart of myocardial damage and arrhythmogenesis due to the toxic effects of elevated intracellular concentrations of the accumulating intermediary metabolites. In this context, long-chain acylcarnitines were known to cause myocardial injury and rhythm disturbances (Corr et al. 1989). Therefore, in the present investigation we evaluated the role of LC3HFA on mitochondrial homeostasis by measuring a wide spectrum of parameters in mitochondria from cardiac muscle of young rats in the hope to better understand the pathophysiology of these diseases. The analysis of mitochondrial respiratory

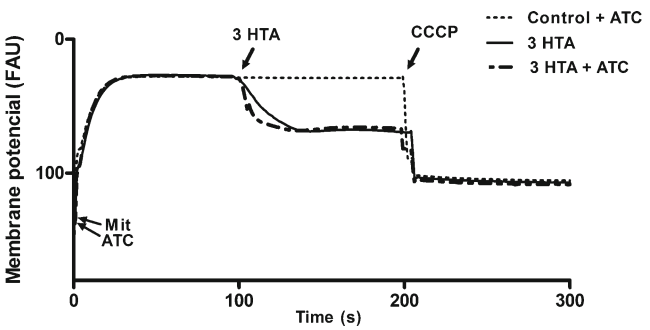

Fig. 8 Effect of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial membrane potential (ΔΨ) using glutamate/malate as substrates. Heart mitochondrial preparations (Mit.; 0.3 mg protein. mL^{-1}) were added to the incubation medium, as indicated by the arrow, in the presence of 5 μM safranine O and 1 μg. mL^{-1} oligomicyn. Atractyloside (ATC, 30 μM) was added in the beginning of the assay, whereas 3 HTA (50 μM) was added at 100 s and CCCP (1 μM) at the end of measurements. The control group did not contain this fatty acid in the medium. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU)

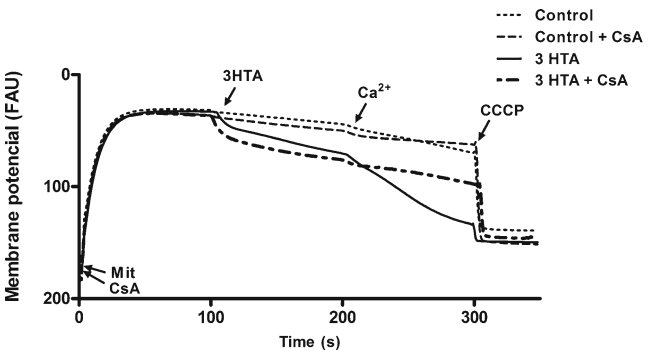

Fig. 9 Effect of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial membrane potential (ΔΨ) using glutamate/malate as substrates. Heart mitochondrial preparations (Mit.; 0.3 mg protein. mL^{-1}) were added to the incubation medium, as indicated by the arrow, in the presence of 5 μM safranine O and 1 μg. mL^{-1} oligomicyn. 3 HTA (10 μM) was added to the incubation medium at 100 s. Ca^{2+} (20 μM) was added to all experiments where indicated by the arrow. Cyclosporin A (CsA, 1 μM) was added in the beginning of the assay, whereas CCCP (1 μM) at the end of measurements. The control group did not contain 3 HTA in the medium. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU)

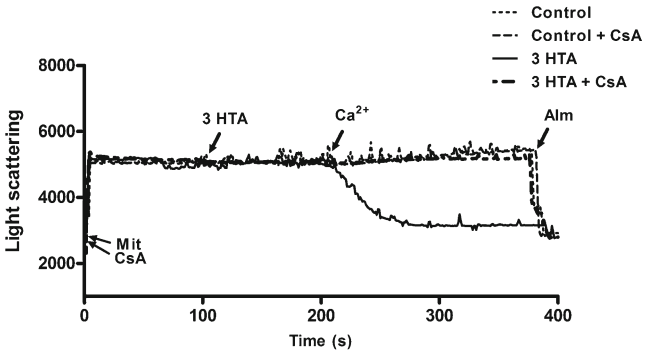

Fig. 10 Effect of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial swelling using glutamate/malate as substrates. Heart mitochondrial preparations (Mit.; 0.4 mg protein. mL^{-1}) and cyclosporin A (CsA, 1 μM) were added to the incubation medium in the beginning of assays. 3 HTA (100 μM) was added to the incubation medium at 100 s. Ca^{2+} (200 μM) was added to all experiments where indicated by the arrow and alamethicin (Alm, 40 μg/mL) at the end of assays. The control group did not contain 3 HTA in the medium. Traces are representative of four independent experiments and were expressed as fluorescence arbitrary units (FAU)

parameters allows the identification of the location of mitochondrial damage, as the phospholipid bilayer permeability, enzyme activities or respiratory substrates/ADP availability (Du et al. 1998). Our findings strongly suggest that the LC3HFA accumulating in MTP and LCHAD deficiencies behave as effective uncouplers of oxidative phosphorylation by a mechanism that seems to be dependent on the carbon chain length. This presumption was based on the results obtained in the various assays showing that the longer the carbon chain the stronger were the effects observed (C_{16}>C_{14}>C_{12}>C_{10}).

Thus, we found that 3HTA and 3HPA significantly increased state 4 respiration and decreased RCR in a dose dependent manner, even at low micromolar concentrations (25 μM), indicating an uncoupling effect on oxidative phosphorylation. The decrease of mitochondrial NAD(P)H content, hydrogen peroxide production and mitochondrial ΔΨ, which were also provoked by 3 HTA and 3 HPA, and to a lesser extent by 3 HDDA, further reinforces the role of these fatty acids as effective uncouplers. The lack of effect of 3 HDDA on state 4 respiration was probably due to the presence of a higher BSA concentration in this assay. Albumin binds fatty acids minimizing their effects on mitochondrial membranes. Moreover, the ADP/O ratio was decreased by 3 HTA, reflecting a less efficient oxidative phosphorylation. The reduction of ADP/O ratio may have occurred due to increased mitochondrial inner membrane permeability leading to proton leak.

3 HTA, at concentrations as low as 10 μM also provoked a significant dissipation of ΔΨ, which was very strong at 50 and 100 μM and could not be further changed by the addition of CCCP, implying a potent action for this LC3HFA. 3 HPA behaved in a similar fashion. We also observed a synergistic effect of 3 HTA and Ca^{2+} decreasing ΔΨ and inducing mitochondrial swelling. It was also shown that the effect of LC3HFA increasing mitochondrial membrane permeability had the involvement of mitochondrial permeability transition since the ΔΨ loss and swelling were prevented by CsA, a classic inhibitor of this process.

Long-chain fatty acids have been considered natural uncouplers of oxidative phosphorylation. The protonophoric mechanism of this effect is thought to be due to their transbilayer movement towards the mitochondrial matrix when undissociated (linked to protons) and their transfer through the inner membrane as anions in the opposite direction. The passage of a dissociated form of fatty acid is usually facilitated by the adenine nucleotide translocator (ANT). Furthermore, the uncoupling potency of fatty acids depends on their chain length and presence of double bonds since the strongest uncoupling effects were found with saturated acids of chain length from 12 to 18 carbon atoms (Schonfeld et al. 1989; Schonfeld 1992; Rottenberg and Hashimoto 1986). The mechanisms by which fatty acids in general are thought to uncouple the oxidative phosphorylation have been related to the involvement of mitochondrial inner membrane anion carriers such as the ANT. This was clearly demonstrated by evidencing that atractyloside abolishes the uncoupling effect induced by low doses of palmitic acid in skeletal muscle mitochondria (Mokhova and Khailova 2005).

Interestingly, under our experimental conditions atractyloside did not prevent the ΔΨ loss induced by 3 HTA, practically ruling out the contribution of ANT in this effect. Therefore, it seems that the mechanisms underlying the uncoupling effects of LC3HFA are different from those provoked by non-hydroxylated long-chain fatty acids. In this regard, LC3HFA may interact with other mitochondrial carriers as the glutamate/aspartate antiporter, with mitochondrial membrane phospholipids, or cause a distortion of the packing of the lipids in the inner mitochondrial membrane leading to alterations in fluidity and ion permeability (Kimelberg and Papahadjopoulos 1974; Lee 1976; Abeywardena et al. 1983; Schönfeld and Struy 1999; Mokhova and Khailova 2005; Skulachev 1998). Furthermore, considering that the LC3HFA induce oxidative stress (Tonin et al. 2010a) and that proton leak is highly correlated to the fatty acid composition of the membrane phospholipids, mitochondrial membrane lipid peroxidation could at least partly explain the uncoupling effects of these fatty acids (Kowaltowski et al. 2009).

On the other hand, we cannot also exclude the possibility that uncoupling of mitochondria may indeed represent a protective and adaptation mechanism leading to a decrease ROS formation (Kowaltowski et al. 2009), as identified in the present study by the reduction of H_2O_2 generation caused by the LC3HFA.

It is difficult to determine at the present the pathophysiological relevance of our present data since the concentrations of the LC3HFA in the heart of patients with MTP and LCHAD deficiencies are unknown. However, it should be stressed that the significant alterations of the biochemical parameters verified in our present study were achieved with small micromolar concentrations (10 μM and higher) of these compounds, similar to those found in plasma of the affected patients (Costa et al. 1998). Furthermore, it is also feasible that the concentrations of the accumulating LC3HFA may dramatically increase in these patients during metabolic crises in which fatty acids are released from the adipose tissue and cannot be metabolized due to the blockage of the enzymatic steps catalyzed by MTP or LCHAD (Costa et al. 1998; Scriver et al. 2001; Halldin et al. 2007). The present data therefore reinforce the hypothesis that accumulation of fatty acids and their derivatives may be involved in the cardiomyopathy characteristically found in these diseases (Ventura et al. 2007) by compromising energy homeostasis.

Conclusions

In conclusion, to the best of our knowledge we give for the first time experimental evidence that the LC3HFA accumulating in MTP and LCHAD deficiencies behave as strong uncoupling agents and decrease the efficiency of oxidative phosphorylation in rat cardiac muscle. Since the phosphorylation state of the ATP pool is very sensitive to small changes in $\Delta\Psi$ (Nicholls 2004) and 3 HTA provoked a marked reduction of $\Delta\Psi$ and mitochondrial swelling through mitochondrial permeability transition, it is feasible that the effects caused by these fatty acids might have deleterious consequences for heart energy homeostasis. Therefore, in case the present in vitro data are confirmed in vivo in animal experiments and also in tissues from patients affected by these fatty acid oxidation defects, it is proposed that mitochondrial dysfunction may contribute to the cardiac alterations characteristic of patients affected by MTP and LCHAD deficiencies. We therefore suggest that mitochondrial permeability transition inhibitors may be potentially interesting as therapeutic candidates for the prevention of heart alterations in these diseases.

Acknowledgements This work was supported by grants from CNPq, PRONEX II, FAPERGS, FAPESP, PROPESQ/UFRGS, FINEP Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00, and Instituto Nacional de Ciência e Tecnologia, Excitotoxicidade e Neuroproteção (INCT-EN).

Conflict of interest statement The authors declare that they have no conflicts of interest.

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CHAPTER IV

Long-chain 3-hydroxy fatty acids accumulating in LCHAD and MTP deficiencies induce mitochondrial permeability transition pore opening in cerebral cortex from young rats

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Scientific article submitted to Molecular Genetics and Metabolism

Long-chain 3-hydroxy fatty acids accumulating in LCHAD and MTP deficiencies induce mitochondrial permeability transition pore opening in cerebral cortex from young rats

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Abstract

Mitochondrial trifunctional protein (MTP) and long-chain 3-hydroxy-acyl-CoA dehydrogenase (LCHAD) deficiencies are inborn errors of fatty acid β-oxidation characterized by accumulation of long-chain 3-hydroxylated fatty acids (LCHFA). Patients present neonatal severe symptoms mostly involving cardiac and hepatic functions, although neurological symptoms are also common. The aim of our present study was to investigate the effects of LCHFA, particularly 3-hydroxytetradecanoic acid (3 HTA), on mitochondrial homeostasis in cerebral cortex of developing rats in order to get insight in the mechanisms of brain damage in these disorders. 3 HTA reduced mitochondrial membrane potential, NAD(P)H content, Ca^{2+} retention capacity and ATP content, as well as induced mitochondrial swelling, cytochrome *c* release and H_2O_2 production. These effects were mainly observed in Ca^{2+}-loaded mitochondria and were prevented by cyclosporine A plus ADP, classical inhibitors of the mitochondrial permeability transition pore (mPTP), and by ruthenium red, a Ca^{+2} uptake blocker. 3-Hydroxydodecanoic and 3-hydroxypalmitic acids that also accumulate in MTP and LCHAD deficiencies also induced mitochondrial swelling and decreased ATP content to a variable degree. The data indicate that these LCHFA are able to induce mPTP opening when mitochondria are loaded with Ca^{2+}, interfering therefore with mitochondrial integrity, energetic production, redox balance and calcium homeostasis. It is proposed that these pathomechanisms may participate in the neurologic dysfunction observed in patients affected by MTP and LCHAD deficiencies.

Keywords: Mitochondrial trifunctional protein deficiency; Long-chain 3-hydroxy acyl-CoA dehydrogenase deficiency; permeability transition pore; calcium

Abbreviations: Alm, Alamethicin; AA, Antimycin A; BSA, bovine serum albumin; CCCP, carbonyl cyanide 3-chlorophenyl hydrazine; CsA, cyclosporin A; EGTA, ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid; FAU, fluorescence arbitrary units; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]; 3 HDA, 3-hydroxydodecanoic acid; 3 HTA, 3-hydroxytetradecanoic acid, 3 HPA 3-hydroxypalmitic acid; LCFA, long-chain fatty acids; LCHAD, long-chain 3-hydroxy-acyl-CoA dehydrogenase; LCHFA, long-chain 3-hydroxy fatty acids; LCKT, long-chain ketoacyl-CoA thiolase; mPTP, mitochondrial permeability transition pore; MTP, mitochondrial trifunctional protein; ROS, reactive oxygen species; RR, ruthenium red.

1. Introduction

Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) is part of the mitochondrial trifunctional protein (MTP) complex that also comprises other two enzyme activities, long-chain enoyl-CoA hydratase and long-chain ketoacyl-CoA thiolase (LCKT). This complex is responsible for mitochondrial oxidation of long-chain fatty acids (LCFA) and an impairment of this pathway, mainly during fasting and prolonged exercise, leads to accumulation of toxic fatty acids, reduction of energy production, decreased acetyl-CoA availability and hypoketosis. Mutations in the genes that encode the MTP complex can lead to reduced or absent activity of all MTP complex enzymes or isolated LCHAD and LCKT deficiencies, being LCHAD deficiency the most frequent disorder.

MTP and LCHAD deficiencies are recognized as severe and life-threatening diseases what makes early identification and treatment essentials to improve patient survival. Both disorders are undistinguishable clinically and biochemically, presenting with multiorgan involvement and elevated levels of long-chain 3-hydroxylated fatty acids (LCHFA) in tissues and biological fluids.

Severe neonatal cardiomyopathy, hepatic dysfunction and skeletal myopathy with rhabdomyolysis are frequently seen in patients with LCHAD and MTP deficiencies. Affected patients can also present a characteristic late onset with irreversible peripheral neuropathy and retinopathy [1], as well as speech and developmental delay that reflects cerebral dysfunction [2]. Since hypoglycemia is commonly found in these individuals, poor glucose uptake, especially during crises of metabolic decompensation could possibly underlie the neurologic symptoms. On the other hand, the long-term neurologic symptomatology has been proposed to be associated with the toxicity of 3-hydroxylong-chain acylcarnitines that accumulate in the patients´ tissues [1]. Although the exact mechanisms of neurotoxicity underlying the toxic effects of the LCHFA are so far poorly established, a recent study

carried out in skin fibroblasts from MTP deficient patients pointed to induction of oxidative stress in these cells [3]. Accordingly, previous *in vitro* studies from our group have shown that LCHFA induce lipid and protein oxidative damage and decrease the antioxidant defenses in rat cerebral cortex [4]. The study also showed impairment in mitochondrial respiration and decrease of reduced equivalents, probably secondary to uncoupling of oxidative phosphorylation in rat forebrain and heart by LCHFA [5, 6]. These data provide evidence that LCHFA disturb energy and redox mitochondrial homeostasis in the brain, and are consistent with the observations that muscle biopsies from LCHAD deficient patients showed abnormal mitochondrial morphology with swollen appearance containing fat infiltration [7].

In this regard, it is presumed that an impairment in mitochondrial bioenergetics can affect mitochondrial calcium homeostasis leading to induction of the mitochondrial permeability transition pore (mPTP) [8-10]. Thus, in the present work we further investigated the role of 3-hydroxydodecanoic (3 HDA), 3-hydroxytetradecanoic (3 HTA) and 3-hydroxypalmitic (3 HPA) acids on brain mitochondrial homeostasis, by evaluating membrane potential, swelling, Ca^{2+} retention capacity, NAD(P)H content, H_2O_2 production, cytochrome *c* release and ATP content in rat mitochondrial preparations from cerebral cortex loaded with Ca^{2+}.

2. Material and methods

2.1 Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of 3 HDA, 3 HTA and 3 HPA were prepared in ethanol (EtOH, 1 % final concentration in the incubation

medium) and added to incubation medium at final concentrations of 10, 30 and 60 μM. The same percentage of EtOH (1%) was present in controls.

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. 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 Principles of Laboratory Animal Care (NIH publication 85-23, revised 1996).

2.3 Preparation of mitochondrial fractions

Mitochondrial preparations from cerebral cortex were isolated according to Rosenthal and co-workers [11], with slight modifications [12]. Animals were decapitated, the cerebral cortex was dissected and homogenized with a glass hand-held homogenizer in ice-cold mitochondria isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1 % bovine serum albumin (BSA, free fatty acid) and 10 mM HEPES, pH 7.2. The homogenate was centrifuged at 2,000 x *g* for 3 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 12,000 x *g* for 8 min at 4°C. The resultant pellet was resuspended in 5 mL of isolation buffer containing 20 μL of 10% digiton, and centrifuged 12,000 x *g* for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 5 mL of isolation buffer without EGTA and centrifuged at 12,000 x *g* for 10 min at 4°C. The final pellet was resuspended in isolation buffer without EGTA in an approximate protein

concentration of 15-20 mg . mL^{-1}. Protein concentration was measured by method of Bradford [13] using BSA as standard. This preparation results in a mixture of synaptosomal and non-synaptosomal mitochondria similar to the general brain composition. The mitochondrial preparations were used in the assays immediately after isolation.

2.4 Standard experimental procedure

Mitochondrial incubations were carried out at 37°C, with continuous magnetic stirring. All spectrofluorimetric assays were conducted in the medium containing 150 mM KCl, 5 mM MgCl2, 30 μM EGTA, 0.1 mg . mL^{-1} BSA, 5 mM HEPES, 2 mM KH2PO4, pH 7.2, using state 4 respiring mitochondria (0.5 mg protein . mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate in the presence of 1 μg . mL^{-1} oligomycin A. 3 HDA (10–60 μM), 3 HTA (10–60 μM), 3 HPA (10–60 μM), $CaCl_2$ (0-30 μM), CCCP (3 μM), antimycin A (AA, 0.1μg . mL^{-1}), alamethicin (Alm, 40 μg . mL^{-1}) were added as indicated by the arrows in the figures. Ruthenium red (RR, 1 μM) and cyclosporin A (CsA, 1 μM) plus ADP (300 μM) when presents, were added in the beginning of the assay. A high Mg^{2+} concentration (5 mM) was used to minimize Ca^{2+} chelation by ADP. The fluorescence/light scattering was monitored on a Hitachi F-2500 spectrofluorometer and data were expressed as fluorescence arbitrary units (FAU). The results shown are representative of three independent experiments.

2.5 Determination of the mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was estimated according to Akerman and Wikstrom [14] and Kowaltowski et al.[15]. The fluorescence of 5 μM cationic dye safranine O, a ΔΨm indicator, was followed at excitation and emission wavelengths of 495 and 586 nm. CCCP was added in the end of measurements to abolish ΔΨm.

2.6 Determination of mitochondrial swelling

Mitochondrial swelling was assayed following the decrease of light scattering at excitation and emission of 540 nm. A decrease in absorbance indicates an increase in mitochondrial swelling. Alamethicin (Alm) was added in the end of the experiment to provoke maximal swelling.

2.7 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. A low concentration of ADP (30 μM) was present in the incubation medium to achieve more consistent mitochondrial Ca^{2+} uptake responses [16]. In the end of the measurements, maximal Ca^{2+} release was induced by CCCP.

2.8 Determination of NAD(P)H fluorescence

Matrix mitochondrial NAD(P)H autofluorescence was measured at 366 nm excitation and 450 nm emission wavelengths. CCCP was added in the end of the measurements to induce maximal NAD(P)H oxidation.

2.9 Mitochondrial hydrogen peroxide (H_2O_2) release

H_2O_2 production was assessed through the oxidation of Ampliflu red (50 µM) in the presence of horseradish peroxidase (0.5 U/mL) [17]. The increase in fluorescence was monitored over time at excitation and emission wavelengths of 563 and 587 nm, respectively. Antimycin A, a respiratory chain inhibitor, was added in the end of the experiments to provoke maximal H_2O_2 production.

2.10 Cytochrome c immunocontent measurement

The swelling experiments were also carried out in the absence of Alm. The incubation medium was collected afterwards and centrifuged at 12,000 x *g* for 10 min at 4°C in order to sediment mitochondria. The resultant pellet was resuspended in 1X RIPA buffer and centrifuged at 10,000 x *g* for 5 minutes at 4°C. The samples were then diluted with Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) and 30 µg of protein/well were fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes with Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad (Hercules, CA, USA). Protein loading and electro-blotting efficiency were verified through Ponceau S staining. The membranes were washed with Tween–Tris buffered saline (Tris 100 mM, pH 7.5, 0.9% NaCl and 0.1% Tween-20) and incubated for 20 minutes at room temperature in SNAP i.d.® 2.0 Protein Detection System Merck Millipore (Billerica, MA, USA) with Cytochrome *c* primary antibody(1:500 dilution range). They were then washed with TTBS. Anti-mouse IgG peroxidase-linked secondary antibody was incubated with the membranes for an additional 20 minutes in SNAP (1:5000 dilution range), washed again and the immunoreactivity was detected by enhanced chemiluminescence using the Supersignal West Pico Chemiluminescent kit from Thermo Scientific (Luminol/Enhancer and Stable Peroxide Buffer). Densitometric analysis of the films was

performed with Image J. software. Blots were developed to be linear in the range used for densitometry. All data were related to total protein.

2.11 ATP quantification

Mitochondrial fractions (0.75 mg protein . mL^{-1}) were incubated in respiring medium containing 0.3 M sucrose, 5 mM MOPS, 5 mM KH_2PO_4, 30 μM EGTA, 0.1 % 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 after 2 min with 1 μg . mL^{-1} oligomycin A. The mitochondrial suspension was then treated with 10 μL of ice-cold 6 M $HClO_4$. After centrifugation at 21,000 x *g* for 5 min at 4 °C, 400 μL aliquots of the supernatant were neutralized with 100 μL of 1 M K_2HPO_4 and submitted to a new centrifugation at 21,000 x *g* for 5 min at 4 °C. ATP was determined in the resulting supernatant by the firefly luciferin–luciferase assay system according to the manufacturer's instructions [18, 19]. The luminescence was measured in a Spectramax M5 microplate spectrofluorimeter. In some experiments oligomycin A was used as a control.

2.12 Statistical analysis

Results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and mean was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the *post-hoc* Tukey'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 GraphPad software.

3. Results

3.1 3 HTA dissipates mitochondrial membrane potential (ΔΨm)

First, we tested the effect of 3 HTA (10 – 60 μM) on mitochondrial membrane potential. We observed that 3 HTA decreases membrane potential in a dose dependent manner (Fig 1A). When mitochondrion was challenged by 15 μM Ca^{2+} in the presence of 30 μM 3 HTA a higher reduction of membrane potential was verified (Fig. 1A). Furthermore, 60 μM 3 HTA provoked itself a marked reduction of ΔΨm. 3 HTA (30 μM)-induced mitochondrial depolarization (decrease ΔΨm) in the presence of Ca^{2+} was prevented by RR, that blocks Ca^{2+} entrance into the mitochondria, and by CsA plus ADP, indicating mPTP induction (Fig. 1B).

3.2 LCHFA provoke mitochondrial swelling

Then, we investigated the influence of 3 HTA on mitochondrial swelling. We verified that 30 μM and higher concentrations of 3 HTA induced an extensive swelling after 30 μM Ca^{2+} addition (Fig. 2A-C). These effects were avoided by RR and by CsA plus ADP when added in the beginning of the assay, reinforcing the involvement of Ca^{2+} and mPTP opening in mitochondrial swelling (Fig. 3). We also showed that 3 HDA and 3 HPA also provoked mitochondrial swelling after Ca^{2+} addition to the mitochondrial preparations whose intensity was dependent on the chain length (Fig. 2D). We verified that the greater effects were achieved the fatty acids with larger carbon chain.

3.3 Mitochondrial Ca^{2+} retention capacity is reduced by 3 HTA

Next, we evaluated the effects of 3 HTA on mitochondrial Ca^{2+} retention capacity since mPTP induction leads to Ca^{2+} release from mitochondria. It was found that 60 μM 3 HTA reduced Ca^{2+}

retention capacity as compared with control mitochondria supported with glutamate plus malate. Furthermore, CsA plus ADP prevented this effect, reinforcing the involvement of mPTP induction in the reduction of mitochondrial Ca^{+2} retention caused by this fatty acid (Fig. 4).

3.4 3 HTA reduces NAD(P)H matrix content

The effect of 3 HTA on NAD(P)H matrix content was also evaluated (Fig 5). We observed that 3 HTA (10-30 μM) induced a decrease of NAD(P)H levels only in the presence of Ca^{2+}, whereas at a higher dose (60 μM) 3 HTA provoked a strong reduction of NAD(P)H independently of Ca^{2+} addition. The data suggest that the reduced equivalents were oxidized or lost from the mitochondrial matrix (Fig. 5A). Furthermore CsA plus ADP and RR prevented the decrease of NAD(P)H content by 30 μM 3 HTA (Fig. 5B).

3.5 3 HTA induces hydrogen peroxide (H_2O_2) production

The previous experiments clearly show the involvement of mPTP in the effects elicited by 3 HTA. Since it is well established that mPTP is usually provoked by oxidative damage, we determined whether mitochondrial H_2O_2 production could be induced by 3 HTA. Fig. 6 shows that 3 HTA significantly increased H_2O_2 generation, especially when Ca^{2+} was loaded to the mitochondrial preparations. It was also observed a full prevention of this effect by CsA plus ADP and by RR when added to the mitochondrial preparations.

3.6 3 HTA induces cytochrome c release

Fig. 7 shows a decrease in cytochrome *c* immunocontent in mitochondria treated with 30 μM 3 HTA and 30 μM Ca^{2+}, reflecting cytochrome *c* release. This effect was prevented by CsA plus ADP

and by RR when added in the beginning of the assay, reinforcing the involvement of mPTP. Alm was used as a positive control once it induces mitochondrial pore formation.

3.7 LCHFA decrease ATP levels

We then tested whether LCHFA could alter mitochondrial ATP levels. We verified that 3 HDA, 3 HTA and 3 HPA decreased ATP content mainly when incubated in the presence of Ca^{2+} (Fig. 8). It can be also seen in the figure that the intensity of this effect was dependent on the fatty acid chain length, so that the longer the carbon chain the greater the effect observed.

4. Discussion

Accumulation of LCHFA occurs in tissues from patients affected by MTP and LCHAD deficiencies. There is some evidence indicating that mitochondrial dysfunction caused by these metabolites may be involved in the acute and chronic neurological symptoms of these diseases [1, 4, 6], although the exact mechanisms of mitochondrial damage are far from understood. Considering that dysregulation of Ca2+ homeostasis is linked to neurodegeneration and generally associated with disturbance of energy and redox homeostasis [9, 20-23], in the present study we searched for alterations of mitochondrial homeostasis caused by 3 HTA and by the other LHCFA accumulating in MTP and LCHAD deficiencies in brain mitochondrial preparations loaded with Ca2+.

We first demonstrated that 3 HTA dissipates mitochondrial membrane potential (ΔΨm) and that this effect was augmented by Ca^{2+} addition. Furthermore, ruthenium red (RR), a potent inhibitor of the mitochondrial Ca^{2+} uptake, prevented this effect, supporting the importance of Ca^{2+} entrance into the mitochondria to provoke the additional dissipation of ΔΨm. Our results also showed that the decrease of ΔΨm elicited by 3HTA after Ca^{2+} addition was due to a non-selective membrane permeabilization caused by mPTP opening, since ΔΨm decrease was accompanied by mitochondrial swelling and both were inhibited by CsA plus ADP. In this particular, CsA inhibits mPTP by binding to cyclophilin D, a mitochondrial matrix protein which has been reported to be a mPTP modulator through its interaction with the adenine nucleotide translocator (ANT) [24-27]. Furthermore, it is known that adenine nucleotides inhibit mPTP through their binding to ANT [16, 28].

Since mitochondrion is important for cellular Ca^{2+} homeostasis [29, 30], we tested mitochondrial Ca^{2+} retention capacity in the presence of 3 HTA. We demonstrated that 3 HTA reduced the mitochondrial Ca^{2+} retention capacity and that this effect was prevented by CsA plus ADP,

providing another indication that mPTP was induced by this fatty acid. Thus, it is conceivable that mPTP opening could allow Ca^{2+} release from the matrix after reaching a threshold overcoming mitochondrial Ca^{2+} retention capacity, as a result of the nonselective inner membrane permeabilization [31-33].

3 HTA also diminished the NAD(P)H matrix content specially after Ca^{2+} loading. Furthermore, the decrease of the reduced equivalents elicited by 3 HTA was inhibited by CsA plus ADP and by RR, implying mPTP induction. Reduction of NAD(P)H matrix content may result from increased NAD(P)H oxidation due to decreased $\Delta\Psi m$ or from its release from the mitochondria through the mPTP formation, potentially leading to impairment of mitochondrial redox homeostasis [34-36].

Regarding to the mechanisms of mPTP induction, it has been shown that fatty acids provoke oxidative stress [4, 37, 38] and uncoupling of oxidative phosphorylation [5, 6, 39, 40], which have been pointed as important mechanisms for mPTP opening [31, 41, 42]. Our results are in line with other studies demonstrating induction of mPTP by uncouplers of oxidative phosphorylation in Ca^{2+}-loaded mitochondria, most probably related to generation of oxygen reactive species and reduction of NAD(P)H content leading to oxidation of membrane protein thiol groups and membrane permeabilization [43-45].

In this regard, we found an induction of H_2O_2 production caused by 3 HTA after Ca^{2+} addition that was prevented by RR and by CsA plus ADP. Therefore, it is possible that mPTP opening provoked by 3 HTA could indirectly result in H_2O_2 production, as previously found in other pathological situations involving reactive oxygen species generation due to mPTP opening [46, 47]. We cannot rule out that 3 HTA-elicited H_2O_2 augment could be due to a reduction of glutathione peroxidase activity. The reduction in NADPH provoked by 3 HTA may impair glutathione reductase activity that converts oxidized glutathione in reduced glutathione that is substrate for glutathione peroxidase.

It is of note that other works have shown that LCFA of saturated carbon chain and also their α,ω- dioic acids can induce nonspecific permeability of the inner membrane in liver mitochondria loaded with Ca^{2+} ions by mechanisms insensitive to CsA, suggesting a diverse mechanism for the LCHFA [48, 49].

3 HTA also reduced mitochondrial cytochrome *c* immunocontent, probably as a consequence of mPTP induction by this compound. In this regard, the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol typically accompanies the osmotic swelling and the physical rupture of the mitochondrial outer membrane provoked by mPTP [50, 51]. Our observations that this effect was prevented by CsA plus ATP and by RR support this hypothesis. In addition, since the release of cytochrome *c* from mitochondria into the cytosol plays a key role in the apoptosis induction by forming the apoptosome complex with Apaf-1 and procaspase-9 and initiating the caspase cascade [52-54], it is conceivable that 3 HTA may induce apoptosis by promoting mPTP in the presence of Ca^{2+}.

Finally we observed a reduction in ATP levels provoked by 3 HTA and by the others LCHFA, reflecting a deleterious consequence to mitochondrial functions induced by these fatty acids. ATP decrease may have occurred due to the uncoupling effect of these fatty acids causing impairment in the maintenance of $\Delta\Psi m$ necessary to ATP biosynthesis [55], although other mechanisms should be investigated. Furthermore, addition of Ca^{2+} to the medium exacerbated this effect, which could be caused by the collapse of $\Delta\Psi m$ and/or loss of adenine nucleotides to the external media through mPTP formation [52, 56].

Our present investigation also showed that 3 HDA and 3 HPA, which also accumulate in LCHAD and MTP deficiencies, provoked mitochondrial swelling and reduction of ATP content, but to a variable degree. We verified that the intensity of their effects on these mitochondrial parameters were dependent on the size of the carbon chain, being the longer molecules more harmful than the shorter.

5. Conclusions

In conclusion, our study provides for the first time evidence that LCHFA act synergistically with Ca^{2+} disrupting mitochondrial energy homeostasis and inducing mPTP in the brain tissue. A permanent pore opening would compromise mitochondrial functions and integrity and lead to impairment of Ca^{2+} homeostasis culminating to apoptotic (or necrotic) cell death [52], which may be associated with brain damage and explain the neurologic alterations presented by patients affected by MTP and LCHAD deficiencies. In this context, there is a growing body of evidence that mPTP opening contributes to tissue degeneration/dysfunction in a variety of diseases and is closely associated to neurodegeneration [57-59].

Acknowledgements

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

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Legend of the figures

Fig. 1. In vitro effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial membrane potential in the presence of Ca^{2+}. (A) 3 HTA (10-60 μM, lines **b-d**) were added 50 seconds after the beginning of the assay to the reaction media containing the mitochondrial preparations (Mit, 0.5 mg protein. mL^{-1} supported by glutamate/malate) and followed by 15 μM Ca^{2+}addition 100 seconds afterwards, as indicated. (B) 30 μM 3 HTA (lines b-d) were added 50 seconds after the beginning of the assay to the reaction media and followed by 15 μM Ca^{2+}addition 100 seconds afterwards, as indicated. Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line **c**) or ruthenium red (RR, 1 μM, line **d**) were added in the beginning of the assay. Controls (line **a**) were performed in the absence of 3 HTA with 15 μM Ca^{2+} addition 150 seconds after the beginning the assay. CCCP (3 μM) was added in the end of the assays. Traces are representative of three independent experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 2. In vitro effects of long-chain 3-hydroxy fatty acids (LCHFA) on mitochondrial swelling in the presence of Ca^{2+}. Increasing concentrations (10-60 μM, lines **b-d**) of (A) 3-hydroxydodecanoic acid (3 HDA), (B) 3-hydroxytetradecanoic acid (3 HTA) or (C) 3-hydroxypalmitic acid (3 HPA) were added 50 seconds after the beginning of the assay in the reaction media containing the mitochondrial preparations (Mit, 0.5 mg protein. mL^{-1} supported by glutamate/malate) and followed by 30 μM Ca^{2+}addition 100 seconds afterwards, as indicated. A comparison of 30 μM 3 HDA, 3 HTA and 3 HPA is depicted in panel (D). Alamethicin (Alm, 40 μg/mg of protein) was added at the end of the measurements. Controls (line **a**) were performed in the absence of 3 HTA with 30 μM Ca^{2+} addition

150 seconds after the beginning the assay. Traces are representative of three independent (animals) experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 3. In vitro effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial swelling in the presence of Ca^{2+}. 30 μM 3 HTA (lines **b-d**) were added 50 seconds after the beginning of the assay in the reaction media containing mitochondrial preparations (Mit, 0.5 mg protein. mL^{-1} supported by glutamate/malate) and followed by 30 μM Ca^{2+}addition 100 seconds afterwards, as indicated. Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line **c**) or ruthenium red (RR, 1 μM, line **d**), when presents, were added in the beginning of the assay. Alamethicin (Alm, 40 μg/mg of protein) was added at the end of the measurements. Controls (line **a**) were performed in the absence of 3 HTA with 30 μM Ca^{2+} addition 150 seconds after the beginning the assay. Traces are representative of three independent (animals) experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 4. In vitro effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial Ca^{2+} retention capacity. Ca^{2+} (30 μM) was added 100 seconds after the beginning of the assay in the reaction media containing the mitochondrial preparations (Mit, 0.5 mg protein. mL^{-1} supported by glutamate/malate), 30 μM ADP and 3HTA (10-60 μM, lines **b-d**), as indicated. Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line **e**) were added in the beginning of the assay. Controls (line **a**) were performed in the absence of 3 HTA with 30 μM Ca^{2+} addition 100 seconds after the beginning the assay. CCCP (3 μM) was added in the end of the assays. Traces are representative of three independent experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 5. In vitro effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial NADH and NADPH content in the presence of Ca^{2+}. (A) 3 HTA (10 μM-60 μM, lines **b-d**) were added 50 seconds after the beginning of the assay to the reaction media containing the mitochondrial preparations (Mit, 0.5 mg protein. mL^{-1} supported by glutamate/malate) and followed by 15 μM Ca^{2+}addition 100 seconds afterwards, as indicated. (B) 30 μM 3 HTA (lines **b-d**) were added 50 seconds after the beginning of the assay to the reaction media and followed by 15 μM Ca^{2+} addition 100 seconds afterwards. Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line **c**) or ruthenium red (RR, 1 μM, line **d**) were added in the beginning of the assay. CCCP (3 μM) was added in the end of the assays, as indicated. Controls (line **a**) were performed in the absence of 3 HTA with 15 μM Ca^{2+} addition 150 seconds after the beginning the assay. Traces are representative of three independent experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 6. In vitro effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial hydrogen peroxide (H_2O_2) production in the presence of Ca^{2+}. 60 μM 3 HTA (line **b**) were added 50 seconds after the beginning of the assay in the reaction media containing the mitochondrial preparations (Mit, 0.5 mg protein. mL^{-1} supported by glutamate/malate) and followed by 30 μM Ca^{2+}addition 100 seconds afterwards, as indicated. Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line **c**) or ruthenium red (RR, 1 μM, line **d**) were added in the beginning of the assay. Controls (line **a**) were performed in the absence of 3 HTA with 30 μM Ca^{2+} addition 150 seconds after the beginning the assay. Antimycin A (AA, 0.1 mg/mL) was added in the end of the measurements. Traces are representative of three independent (animals) experiments and were expressed as fluorescence arbitrary units (FAU).

Fig. 7. In vitro effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial cytochrome *c* immunocontent. 30 μM 3 HTA were added 50 seconds after the beginning of the assay in the reaction media containing the mitochondrial preparations (0.5 mg protein. mL^{-1} supported by glutamate/malate) and followed by 30 μM Ca^{2+} addition 100s afterwards. Controls were performed in the absence of 3 HTA with 30 μM Ca^{2+} addition 150 seconds after the beginning the assay. Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) and ruthenium red (RR, 1 μM), when presents, were added in the beginning of the assay. Alamethicin (Alm, 40 μg/mg of protein) was used as a positive control. Total protein was used as loading control. Values are means ± standard deviation for three independent experiments expressed as relative intensity. *P < 0.05 **P < 0.01, ***P < 0.001 compared to control (Tukey's multiple comparison test).

Fig. 8. In vitro effects of long-chain 3-hydroxy fatty acids (LCHFA) on ATP content in mitochondrial preparations (Mit, 0.5 mg protein. mL^{-1} supported glutamate/malate). 3-hydroxydodecanoic acid (3 HDA), 3-hydroxytetradecanoic acid (3 HTA) and 3-hydroxypalmitic acid (3 HPA) were added in the beginning of the assay. 30 μM Ca^{2+}, when present, were added 90 seconds afterwards. Controls were performed in the absence of LCHFA with 30 μM Ca^{2+} addition 150 seconds after the beginning the assay. Oligomycin A (Oligo, 1 μM. 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^{-1}). *P < 0.05 **P < 0.01, ***P < 0.001 compared to controls (Tukey's multiple comparison test).

Figure 1.

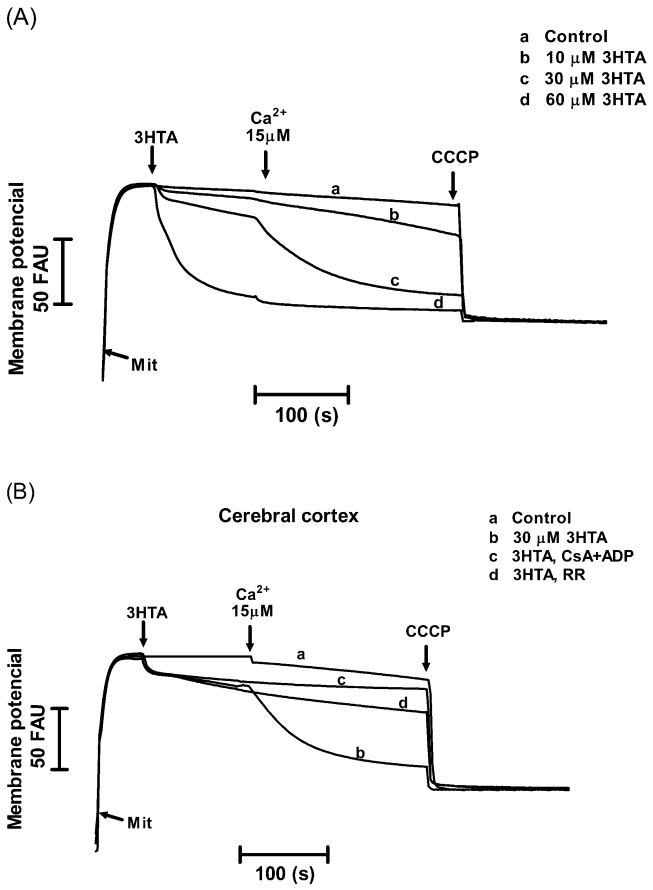

(A)
a Control
b 10 μM 3HTA
c 30 μM 3HTA
d 60 μM 3HTA
3HTA
Ca2+ 15μM
CCCP
a
b
c
d
Membrane potencial
50 FAU
Mit
100 (s)
(B)
Cerebral cortex
a Control
b 30 μM 3HTA
c 3HTA, CsA+ADP
d 3HTA, RR
3HTA
Ca2+ 15μM
CCCP
a
c
d
b
Membrane potencial
50 FAU
Mit
100 (s)

Figure 2.

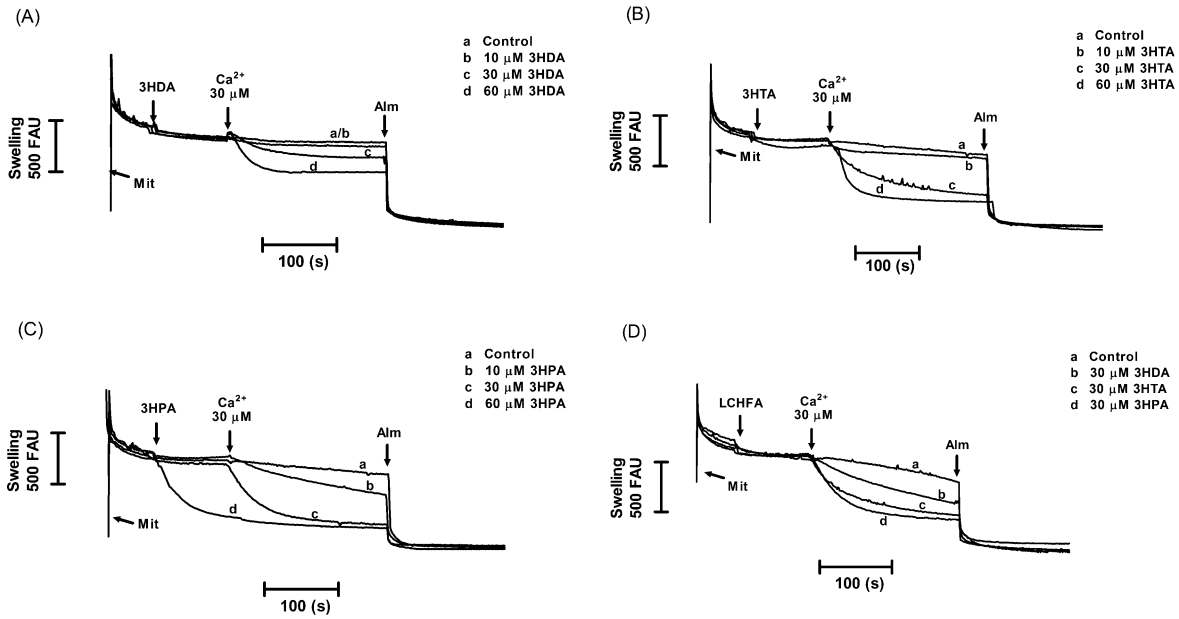

(A)
a Control
b 10 μM 3HDA
c 30 μM 3HDA
d 60 μM 3HDA
3HDA
Ca2+ 30 μM
Alm
a/b
c
d
Mit
Swelling 500 FAU
100 (s)
(B)
a Control
b 10 μM 3HTA
c 30 μM 3HTA
d 60 μM 3HTA
3HTA
Ca2+ 30 μM
Alm
a
b
c
d
Mit
Swelling 500 FAU
100 (s)
(C)
a Control
b 10 μM 3HPA
c 30 μM 3HPA
d 60 μM 3HPA
3HPA
Ca2+ 30 μM
Alm
a
b
c
d
Mit
Swelling 500 FAU
100 (s)
(D)
a Control
b 30 μM 3HDA
c 30 μM 3HTA
d 30 μM 3HPA
LCHFA
Ca2+ 30 μM
Alm
a
b
c
d
Mit
Swelling 500 FAU
100 (s)

Figure 3.

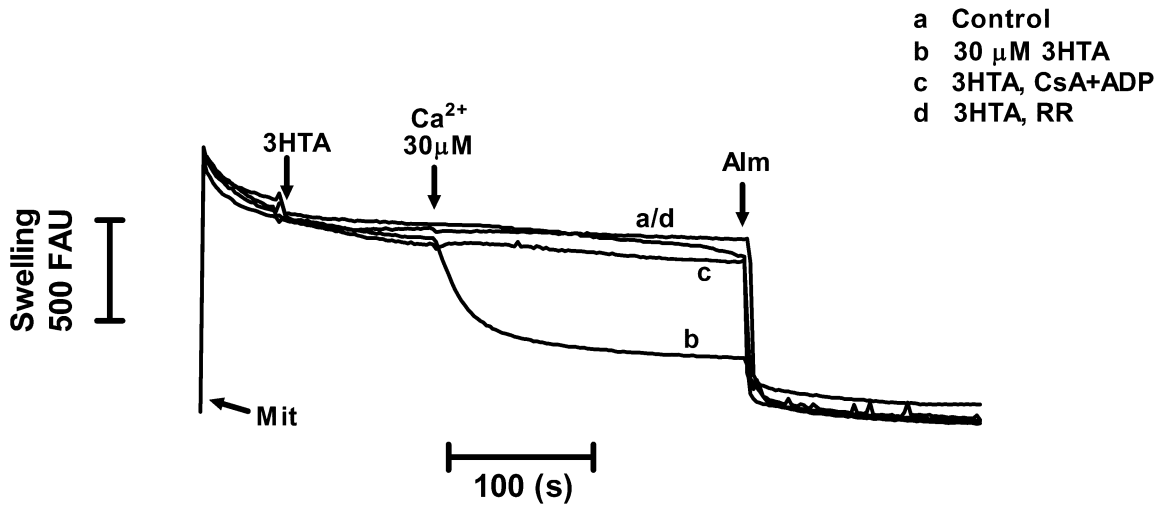

a Control
b 30 μM 3HTA
c 3HTA, CsA+ADP
d 3HTA, RR
3HTA
Ca2+
30μM
Alm
a/d
c
b
Swelling
500 FAU
Mit
100 (s)

Figure 4.

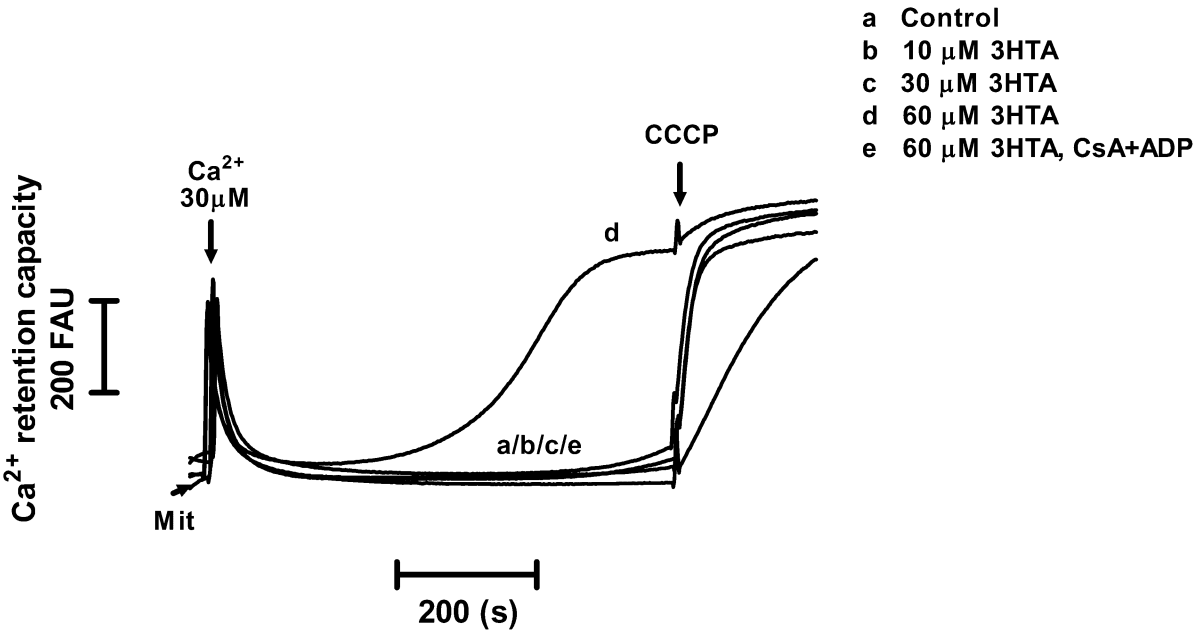

a Control
b 10 μM 3HTA
c 30 μM 3HTA
d 60 μM 3HTA
e 60 μM 3HTA, CsA+ADP
CCCP
Ca2+
30μM
d
a/b/c/e
Mit
Ca2+ retention capacity
200 FAU
200 (s)

Figure 5.

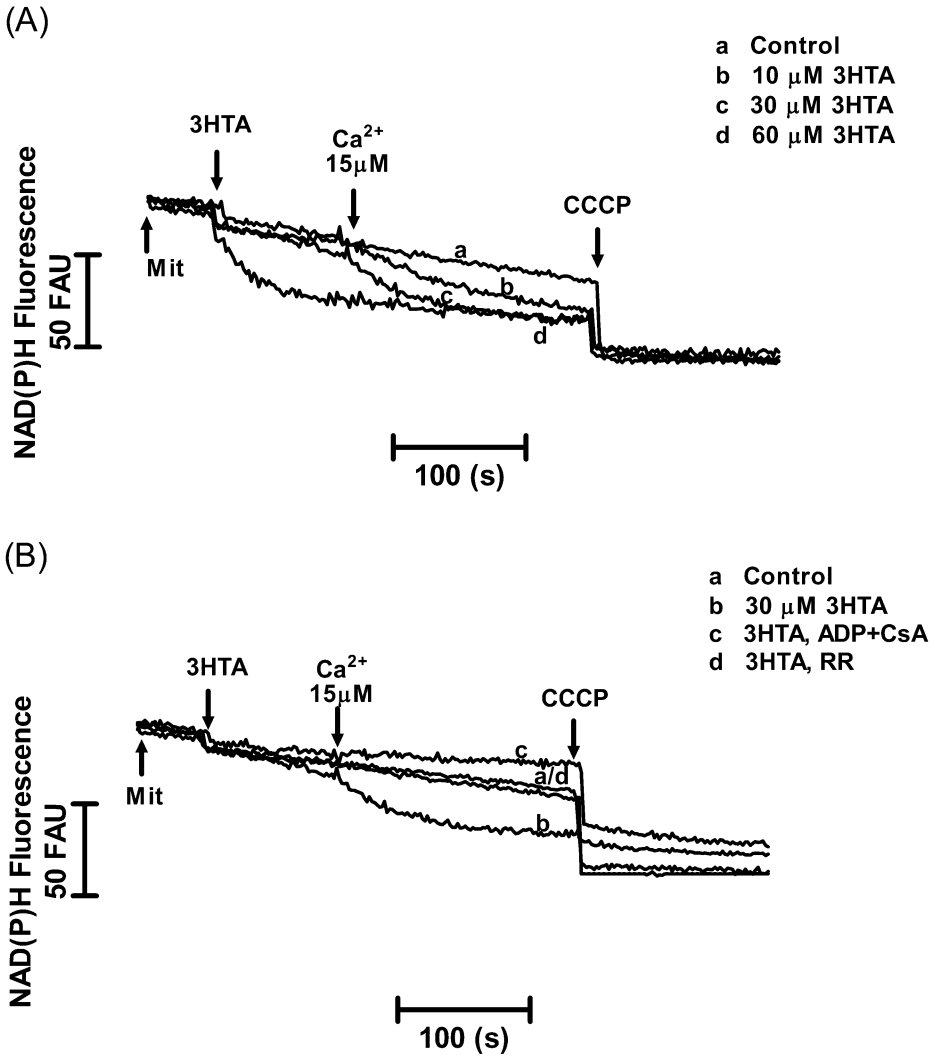

(A)
a Control
b 10 μM 3HTA
c 30 μM 3HTA
d 60 μM 3HTA
3HTA
Ca2+
15μM
CCCP
Mit
a
b
c
d
NAD(P)H Fluorescence
50 FAU
100 (s)
(B)
a Control
b 30 μM 3HTA
c 3HTA, ADP+CsA
d 3HTA, RR
3HTA
Ca2+
15μM
CCCP
Mit
c
a/d
b
NAD(P)H Fluorescence
50 FAU
100 (s)

Figure 6.

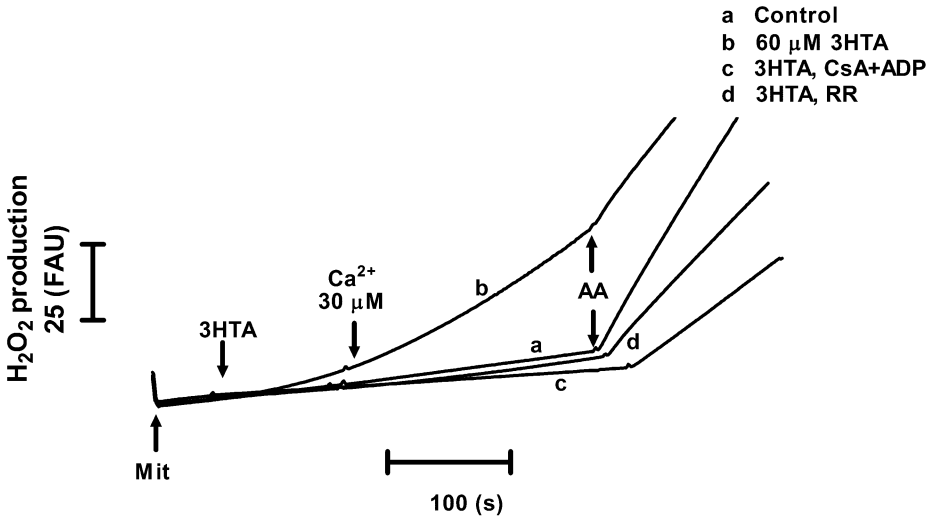

a Control
b 60 μM 3HTA
c 3HTA, CsA+ADP
d 3HTA, RR
H2O2 production
25 (FAU)
3HTA
Ca2+
30 μM
b
AA
a
d
c
Mit
100 (s)

Figure 7.

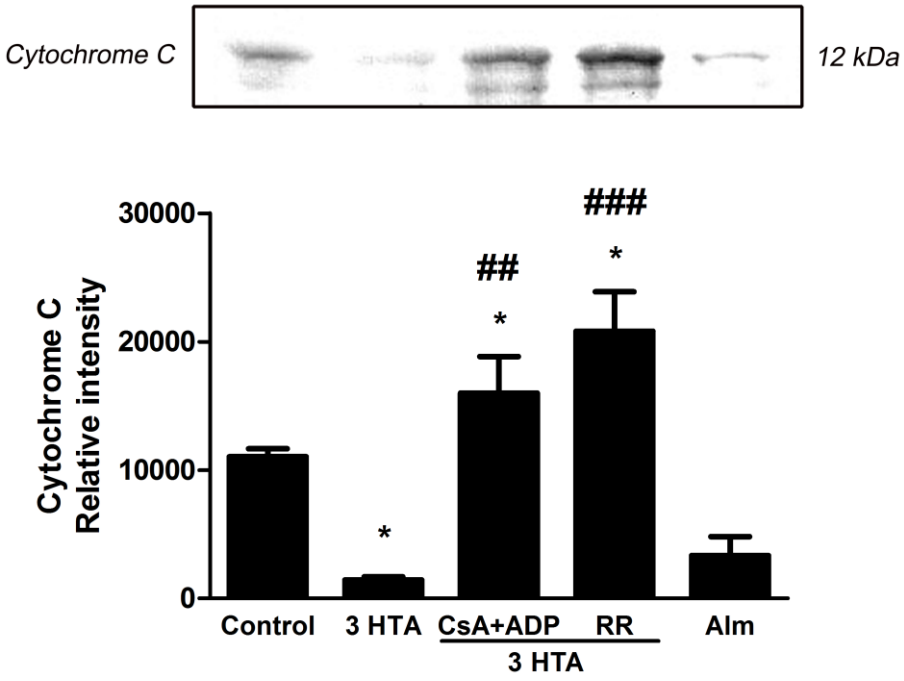
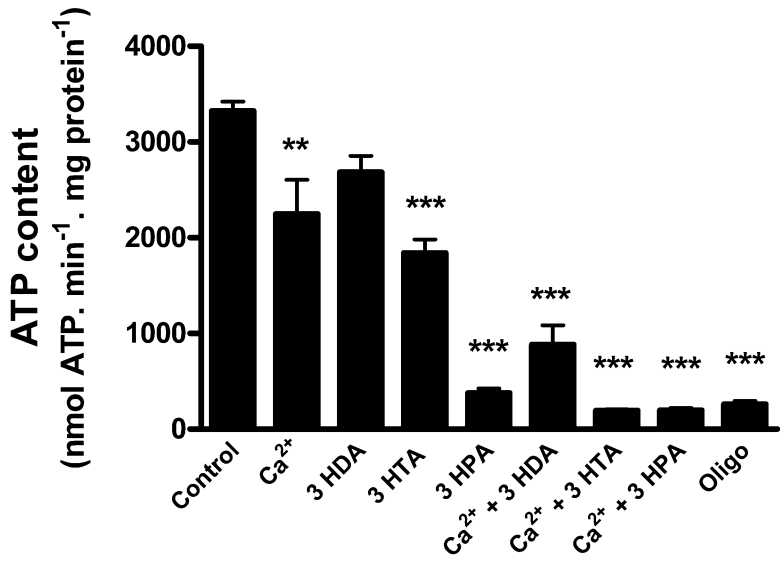

Cytochrome C
12 kDa
Cytochrome C
Relative intensity
30000
20000
10000
0
*
##
*
###
*
Control
3 HTA
CsA+ADP
RR
3 HTA
Alm

Figure 8.


ATP content
(nmol ATP. min-1 . mg protein-1)
4000
3000
2000
1000
0
**
***
***
***
***
***
Control
Ca2+
3 HDA
3 HTA
3 HPA
Ca2+ + 3 HDA
Ca2+ + 3 HTA
Ca2+ + 3 HPA
Oligo

CHAPTER V

Long-chain 3-hydroxylated fatty acids do not induce oxidative stress in heart of rats *in vitro*

Additional results

1. Material and methods

1.1 Animals and reagents

Thirty-day-old male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS – Brazil, were used. The animals were maintained on a 12:12 h light / dark cycle (lights on 07.00 - 19.00 h) in air conditioned constant temperature (22°C ± 1°C) colony room, with free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil and followed the "Principles of Laboratory Animal Care (NIH publication 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of 3-hydroxydocecanoic acid (3 HDA), 3-hydroxytetradecanoic acid (3 HTA) and 3-hydroxypalmitic acid (3 HPA) were prepared in ethanol (1 % final concentration in the incubation medium) and added to incubation medium at final concentrations of 10 μM, 25 μM, 50 μM and 100 μM. The same amount of ethanol (EtOH, 1%) was present in controls.

1.2 Tissue preparation and incubation

The rats were deeply anesthetized with ketamine plus xilazine (75 and 10 mg/kg i.p., respectively) and were submitted to intracardiac perfusion with saline (NaCl 0.9 %). The heart was removed, weighed, and homogenized in 10 volumes (1:5, w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 1,000 g for 20 min at 4 ^{o}C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and aliquots were incubated

for 1 h at 37 ^{o}C with the fatty acids 3 HDA, 3HTA and 3 HPA at concentrations ranging from 10 to 100 μM. Controls did not contain any of these metabolites in the incubation medium. After incubation, the aliquots were used to determine thiobarbituric acid-reactive substances (TBA-RS), carbonyl formation, sulfhydryl oxidation, and glutathione (GSH) concentrations.

We always carried out parallel experiments with blanks (controls) in the presence or absence of the tested metabolites and without heart supernatants in order to detect artifacts caused by the LCHFA in the assays.

1.3 Determination of thiobarbituric acid-reactive substances (TBA-RS) levels

Thiobarbituric acid-reactive substances (TBA-RS) levels TBA-RS levels were assayed according to the method described by Yagi (1998) with slight modifications. Two volumes of 10 % trichloroacetic acid (TCA) and one volume of 0.67 % TBA in 7.1 % sodium sulfate were added to tissue supernatants (0.3–0.5 mg of protein) for 1 h in a boiling water bath. The mixture was allowed to cool on water for 5 min. The resulting pink stained complex was extracted with 400 μL butanol. Fluorescence of the organic phase was read at 515 and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/mg protein and the results were expressed as percentage of control.

1.4 Determination of protein carbonyl formation

Protein carbonyl formation, a marker of oxidized proteins, was measured spectrophotometrically according to Reznick and Packer (1994). Two hundred microliters of heart supernatants (0.3–0.5 mg of protein) were treated with 400 μL of 10 mM 2,4-dinitrophenylhydrazine

(DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 h at room temperature. Samples were then precipitated with 20 % TCA and centrifuged for 5 min at 9,000 *g*. The pellet was then washed with ethanol:ethyl acetate (1:1, v/v), and re-dissolved in 550 μL of 6M guanidine prepared in 2.5 N HCl. The difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl content determined at 370 nm. The results were calculated as nmol of carbonyl groups/mg of protein, using the extinction coefficient of 22,000 x 10^6 nmol/mL for aliphatic hydrazones and expressed as percentage of control.

1.5 Determination of sulfhydryl (thiol) oxidation

This assay is based on the reduction of 5,50-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB), the absorption of which is measured spectrophotometrically (Aksenov and Markesbery, 2001). Briefly, 0.1 mM DTNB was added to 50 μL of heart supernatant (0.3–0.5 mg of protein), followed by an incubation of 30 min at room temperature in a dark room. Absorption was measured at 412 nm. Results were calculated as nmol TNB/mg protein and expressed as percentage of control.

1.6 Determination of reduced gluthatione (GSH) concentrations

Reduced glutathione (GSH) concentrations were measured according to (Browne and Armstrong, 1998) with slight modifications. After the incubation, one volume of metaphosphoric acid was added to the sample, which was then centrifuged for 10 min at 7,000 *g*. One hundred and eighty-five microliters of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and 15 μL of o-phthaldialdehyde (1 mg/mL) were added to 30 μL of heart supernatants (0.3–0.5 mg of protein). This mixture was then incubated at room temperature in a dark room for 15 min. Fluorescence was

measured by excitation and emission wavelengths of 350 and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.001–1 mM) and the concentrations were calculated as nmol/mg protein and expressed as percentage of control.

1.7 Protein determination

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

1.8 Statistical analysis

Results are presented as mean ± standard deviation of the mean. Assays were performed in triplicate and the mean was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA). All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

2. Results

2.1 *LCHFA were not able to induce lipid peroxidation in heart rat*

We initially tested the *in vitro* influence of LCHFA on TBA-RS levels, a lipid oxidation marker, in heart rat. Figure 1 shows that 3 HDA, 3 HTA and 3 HPA are not able to alter TBA-RS levels (Figure 1).

2.2 *LCHFA did not induce protein oxidative damage in heart rat*

Next, we evaluated the effect of LCHFA on carbonyl formation and sulfhydryl oxidation in order to evaluate protein oxidation parameters. We found that 3 HDA, 3 HTA and 3 HPA did not alter either protein carbonyl formation or decreased sulfhydryl content (Figure 2 and 3, respectively).

2.3 *GSH concentrations were not alter reduced gluthatione (GSH) levels*

Finally, we tested the *in vitro* effect of 3 HDA, 3 HTA and 3 HPA on the concentrations of GSH, the most important brain antioxidant defense. We found that the LCHFA had no effect on GSH levels (Figure 4).

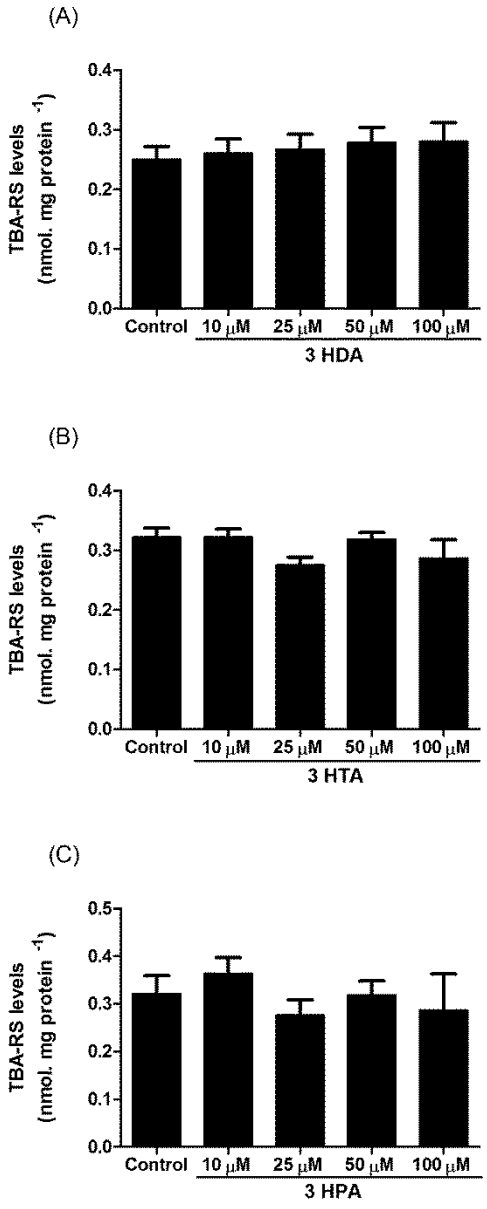


Fig. 1 *In vitro* effect of long-chain hydroxyl fatty acids on thiobarbituric acid reactive substances (TBA-RS) levels in rat heart. Heart supernatants were incubated in the presence of 3-hydroxydodecanoic acid (3 HDA, A); 3-hydroxytetradecanoic acid (3 HTA, B) and 3-hydroxypalmitic acid (3 HPA, C) at concentrations ranging from 10 – 100 μM. Values are means ± standard deviation

for six independent experiments (animals) performed in triplicate and are expressed as nmol/mg protein.

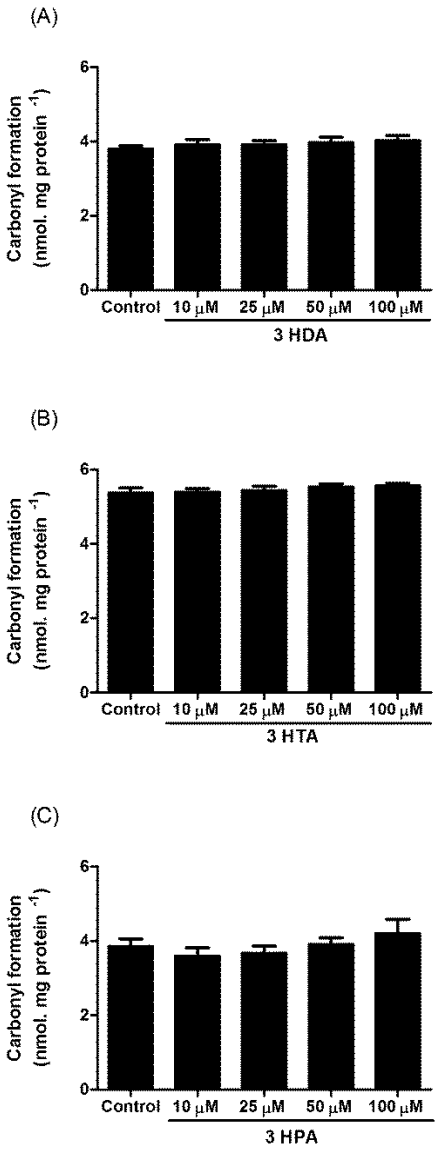


Fig. 2 *In vitro* effects of long-chain hydroxyl fatty acids on carbonyl formation in rat heart. Heart supernatants were incubated in the presence of 3-hydroxydodecanoic acid (3 HDA, A); 3-hydroxytetradecanoic acid (3 HTA, B) and 3-hydroxypalmitic acid (3 HPA, C) at concentrations

ranging from 10 – 100 μM. Values are means ± standard deviation for six independent experiments (animals) performed in triplicate and are expressed as nmol/mg protein.

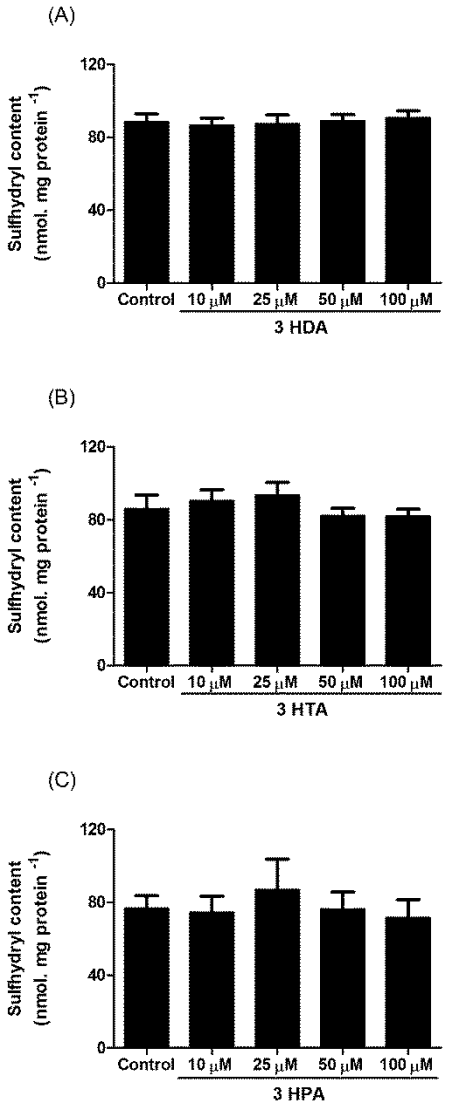


Fig. 3 *In vitro* effects of long-chain hydroxyl fatty acids on sulfhydryl oxidation in rat heart. Heart supernatants were incubated in the presence of 3-hydroxydodecanoic acid (3 HDA, A); 3-hydroxytetradecanoic acid (3 HTA, B) and 3-hydroxypalmitic acid (3 HPA, C) at concentrations

ranging from 10 – 100 μM. Values are means ± standard deviation for six independent experiments (animals) performed in triplicate and are expressed as nmol/mg protein.

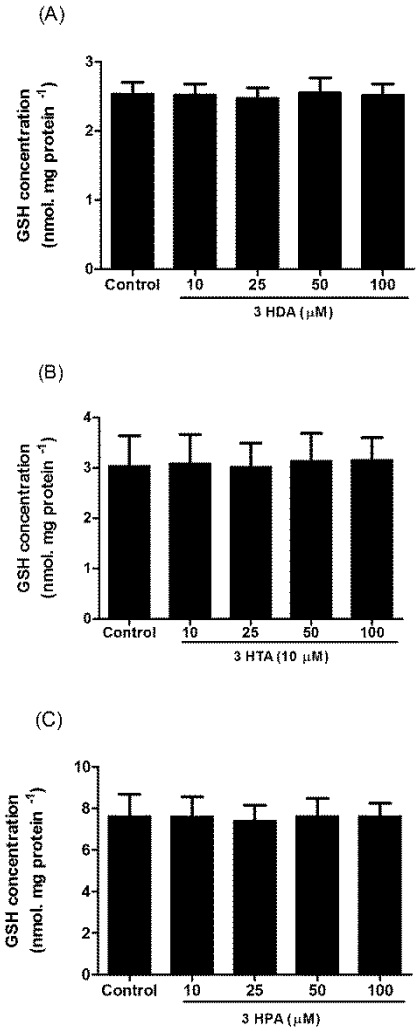


Fig. 4 *In vitro* effects of long-chain hydroxyl fatty acids on reduced glutathione (GSH) concentration in rat heart. Heart supernatants were incubated in the presence of 3-hydroxydodecanoic acid (3 HDA, A); 3- hydroxytetradecanoic acid (3 HTA, B) and 3-hydroxypalmitic acid (3 HPA, C) at concentrations

ranging from 10 – 100 μM. Values are means ± standard deviation for six independent experiments (animals) performed in triplicate and are expressed as nmol/mg protein.

SECTION III -

DISCUSSION AND CONCLUSIONS

3. Discussion

The interest in inherited defects of fatty acid oxidation (FAO) has been growing in the last two decades because the incidence of these disorders is relatively high and some therapeutic measures can be taken to avoid morbidity and mortality (Spiekerkoetter et al., 2003). The major clinical manifestations occur in heart and skeletal muscles, liver and brain that are tissues highly dependent on energy. In this context, symptoms are mainly manifested during illnesses leading to catabolic states or after prolonged fasting, situations in which fatty acid oxidation is activated (Eaton et al., 1996).

MCAD and LCHAD deficiencies are relatively frequent FAO disorders having an incidence of approximately one in 14,000 and one in 100,000 births, respectively. Because MCAD deficiency has the same incidence of PKU and preventive measures avoid symptomatology, it has been included in neonatal mass screening programs during the last few years in many centers.

The clinical phenotype of these disorders has been attributed to energy deficiency provoked by a blockage in the oxidation of fatty acids and by hypoketotic hypoglycemia. However, symptoms usually precede the profound hypoglycemia suggesting toxic effects the accumulating metabolites. Furthermore, a growing body of evidence has shown that the metabolites accumulating in FAO disorders present toxic effects in brain, heart, skeletal muscle and liver (de Assis et al., 2006; de Assis et al., 2003; Scaini et al., 2012; Schuck et al., 2007; Schuck et al., 2010; Schuck et al., 2009a; Tonin et al., 2013; Tyni et al., 1996; Ventura et al., 2005; Ventura et al., 1996; Ventura et al., 2007) supporting a toxic effects of these compounds. However, considering that the exact pathological mechanisms underlying this toxicity are still far from understood, in the present work we searched for the molecular

mechanisms behind the toxicity of FA and their carnitine derivatives accumulated in MCAD, MTP and LCHAD deficiencies that are involved in the heart and brain damage in these disorders. We initially investigated the role of the medium-chain acylcarnitines (MCAC) that accumulate in MCAD deficiency and the 3-hydroxy-fatty acids accumulating in LCHAD and MTP deficiencies on redox and energy metabolism in brain and heart of young rats. For this purpose, we used different *in vitro* preparations, such as homogenates and mitochondrial fractions from rat heart and cerebral cortex, as well as cultured skin fibroblasts from MCAD and MTP deficient patients.

We should consider that the brain is more susceptible to oxidative damage compared to other organs because of its biochemical composition. Cerebral tissue contains high amounts of unsaturated lipids (labile to peroxidation), higher concentrations of iron in certain brain regions and poor antioxidant defenses having about 10% of the antioxidant activity as that of the liver (Floyd and Carney, 1992). Thus, considering that oxidative stress has been pointed as a contributing factor of tissue damage in various neurodegenerative diseases and that the brain is particularly susceptible to oxidative stress, in the first chapter of this Thesis, we investigated the role of MCAC on parameters of oxidative stress in cerebral cortex of young rats.

We first observed that hexanoylcarnitine (HC), *cis*-4-decenoylcarnitine (cDC) and particularly decanoylcarnitine (DC) provoked marked increase of lipid peroxidation probably through formation of peroxyl and hydroxyl radical once trolox (TRO) and melatonin (MEL) prevented these effects. Moreover, DC induced protein oxidation evidenced by increased carbonyl formation and decreased sulfhydryl content. We cannot exclude the possibility that aldehydes resulting from lipid peroxidation may have also induced carbonyl generation (Dalle-Donne et al., 2003). Considering that protein oxidative damage usually leads to protein dysfunction since many oxidized proteins lose their function (Dalle-Donne et al., 2009). Our present results may possibly underlie at least in part the deleterious

effects of the compounds on cell membranes which are enriched of lipids and by inactivating crucial proteins essential for cell functioning. We also verified that HC, DC and cDC markedly decreased brain GSH concentrations indicating that rat cortical antioxidant defenses were severely compromised by the MCAC and especially by DC, which exerted the most prominent effect. Similarly to the TBA-RS values, TRO and MEL were able to prevent DC-induced decrease of GSH levels in cerebral cortex, reinforcing the role of the highly toxic hydroxyl radical and peroxyl radicals in the reduction of GSH concentrations provoked by DC. Moreover, we evidenced a strong inverse correlation between TBA-RS increase and GSH decrease caused by DC, indicating that the decrease of the brain antioxidant defenses and the induction of lipid peroxidation are dependent effects probably caused by increase reactive species generation. Once L-carnitine itself did not alter any of the evaluated parameters it is presumed that these effects were not due to the carnitine component of their molecules. Furthermore, our results also showed that the hexanoic acid and hexanoylglycine were not able to induce lipid (TBA-RS increase), protein (sulfhydryl decrease) oxidative damage, or decrease the brain antioxidant defenses (GSH reduction), suggesting that carnitine derivatives of MCFA may be more toxic than the free fatty acids accumulating in MCAD deficiency.

Although plasma MCAC levels encountered in MCAD-deficient patients are lower than the concentrations used in the present study, we emphasize that during crises of acute encephalopathy the free MCFA concentrations may increase dramatically due to accelerated catabolism. In conclusion, in the first part of our work we report for the first time that the MCAC accumulating in MCAD deficiency provoke oxidative stress in cerebral cortex of developing rats. Considering previous data showing that the free MCFA, from which the acylcarnitines originate, also induce lipid and oxidative damage in the brain (Schuck et al., 2007; Schuck et al., 2009a), it is tempting to speculate that excessive free radicals might contribute, at least in part, to the neuropathology of this disorder, especially during episodes of

metabolic stress in which the tissue concentrations of the accumulating metabolites increase dramatically.

Preview reports have shown that cultured fibroblasts from FAO defects and especially MTP deficient patients are vulnerable to oxidative stress (Zolkipli et al., 2011), possibly strengthening the importance of our *in vitro* animal data demonstrating that oxidative damage is caused by metabolites accumulating in MCAD and MTP/LCHAD deficiencies (Scaini et al., 2012; Schuck et al., 2007; Schuck et al., 2009a; Tonin et al., 2010b).

In the present study we investigated superoxide production and cell death in fibroblasts from MCADD and MTPD patients cultured under different metabolic conditions, using standard medium (11 mM glucose) or medium without glucose supplemented by 11 mM galactose (Gal) or by 11 mM galactose plus 100 μM palmitate (GalPalm) (metabolic stress conditions).

Under standard growing conditions, fibroblasts from MTPD patients presented higher levels of superoxide and cell death compared to controls, whereas these parameters were not altered in MCADD fibroblasts. Furthermore, metabolic stress conditions caused a further increase in the amount of stressed cells (higher superoxide levels) in fibroblasts from MTPD patients, as compared to standard cultured conditions, and a significant increase of the levels of this free radical in MCADD patients with no cell death. Furthermore, under metabolic stress conditions, superoxide levels were similarly increased in fibroblasts from MCADD and MTPD patients. In contrast, cells from patients submitted to metabolic stress had a similar rate of cell death comparing to those observed in basal conditions. These observations may indicate that fibroblasts from MCADD and MTPD patients are chronically stressed and the establishment of stress conditions did not accentuate cell death. There was also no difference between the stress conditions (Gal or GalPalm) and the time of exposure (12 or 24 h) regarding

superoxide levels and cell mortality. These data suggest that a chronic cell exposure to oxidative stress that may be related to cell death at least in MTPD patients since oxidative damage represents a common mechanism underlying cell collapse. In this context, our data showing a significantly positive correlation between the sytox intensity (cell death) and MitoSOX levels (superoxide levels) in fibroblasts from these patients is in accordance with this presumption.

Moreover, no correlation between the parameters evaluated (superoxide levels and cell death) with the distinct mutations presented by the patients affected by both disorders was found, implying a lack of correlation between genotype and the parameters examined in this study.

Taken together, we showed strong evidences of ROS induction in some FAO disorders, particularly MCAD and MTP deficiencies. We cannot, however, rule out that other mechanisms might take part in the tissue damage characteristic of these disorders. In this context, the observations of lactic acidemia, inhibition of various complexes of the respiratory chain and mitochondrial morphological abnormalities in patients affected by MTP and LCHAD deficiencies point to a compromised mitochondrial function (Das et al., 2000; Spiekerkoetter et al., 2008; Tyni et al., 1996; Ventura et al., 1998). Furthermore, it has been postulated that accumulation of LCFA and their derivatives (CoA esters and carnitines), including the LCHFA, may represent a major contributing factor for the clinical and biochemical phenotypes in long-chain FAO deficient patients, by causing impairment of energy homeostasis (Ventura et al., 2007). Our recent findings showing that LCHFA induce oxidative stress and disrupt mitochondrial energy homeostasis in rat brain support this hypothesis (Tonin et al., 2010a; Tonin et al., 2010b). Thus, we dedicated chapters III, IV and V to the investigation of the pathogenic mechanisms involved in the cardiomyopathy and brain damage presented by MTP and LCHAD deficient patients.

Pathogenetic mechanisms involved in the development of cardiomyopathy in patients with MTP and LCHAD deficiencies are poorly understood, although inadequate energy supply, presence of misfolded proteins and toxicity of the accumulating LCHFA may play a role. In this context, it was shown that long-chain acylcarnitines cause myocardial injury and rhythm disturbances (Corr et al., 1989). Therefore, in the present investigation we evaluated the role of LCHFA on mitochondrial homeostasis by measuring a wide spectrum of bioenergetics parameters in mitochondria from cardiac muscle of young rats in the hope to better understand the pathophysiology of these diseases.

First, we found that 3-hydroxytetradecanoic (3 HTA) and 3-hydroxypalmitic (3 HPA) acids act as uncouplers of oxidative phosphorylation, as evidenced by increased state 4 respiration and decreased respiratory control ratio in heart mitochondrial preparations. Moreover, the observed decrease in mitochondrial matrix pool of NAD(P)H, the reduction in hydrogen peroxide production and mainly the drop in mitochondrial $\Delta\Psi$ provoked by these fatty acids further reinforces the role of these fatty acids as effective uncouplers of oxidative phosphorylation in this organ. We also observed that the stronger effects were observed with the longer carbon chain of these compounds. Furthermore, the ADP/O ratio was decreased by 3 HTA, reflecting a less efficient oxidative phosphorylation probably due to increased mitochondrial inner membrane permeability causing proton leak. These findings strongly suggest that the LCHFA accumulating in MTP and LCHAD deficiencies uncouple the oxidative phosphorylation by a mechanism that seems to be dependent on the carbon chain length. We also observed that 3 HTA induced a marked $\Delta\Psi$ depolarization and mitochondrial swelling in Ca^{2+} loaded mitochondria. Such effects were prevented by cyclosporin A, a classic inhibitor of the membrane permeability transition pore (mPTP), suggesting the involvement of the mPTP opening in this process.

Although the adenine nucleotide translocator (ANT) are thought to participate in the uncoupling effect of LCFA in mitochondria from liver and skeletal muscle (Andreyev et al., 1989), under our experimental conditions atractyloside, an ANT blocker, did not prevent the $\Delta\Psi$ loss induced by 3 HTA, ruling out the contribution of ANT in this effect in cardiac preparations. Thus, it seems that the mechanisms underlying the uncoupling effects of LCHFA are different from those provoked by non-hydroxylated LCFA. So, it is feasible that LCHFA may interact with other mitochondrial carriers as the glutamate/aspartate antiporter, with mitochondrial membrane phospholipids, or cause a distortion of the packing of the lipids in the inner mitochondrial membrane leading to alterations in fluidity and ion permeability (Abeywardena et al., 1983; Kimelberg and Papahadjopoulos, 1974; Lee, 1976; Mokhova and Khailova, 2005; Schönfeld and Struy, 1999; Skulachev, 1998).

Regarding to the mechanisms responsible for brain dysfunction in MTP and LCHAD deficiencies, there are also some evidences indicating mitochondrial dysfunction caused by the accumulating metabolites, suggesting therefore the involvement of these compounds in the neurological symptoms of the affected patients (Spiekerkoetter et al., 2004; Tonin et al., 2010a; Tonin et al., 2010b). Thus, the previous chapters directed our study to the evaluation of the effects of accumulating metabolites in MTP and LCHAD on mitochondrial energetic and redox homeostasis, as well as mitochondrial membrane integrity and calcium metabolism. Considering that dysregulation of Ca^{2+} homeostasis is linked to neurodegeneration and generally associated with disturbance on mitochondrial function (Gandhi et al., 2009; Jaiswal et al., 2009; Martin et al., 2009; Quintanilla and Johnson, 2009; Rosenstock et al., 2004), in chapter IV of the Thesis, we searched for alterations of

mitochondrial homeostasis caused by 3 HTA and by the other LHCFA accumulating in these FAO diseases in mitochondrial preparations obtained from cerebral cortex and loaded with Ca^{2+}.

We demonstrated that 3 HTA dissipates ΔΨ and diminishes the NAD(P)H matrix content specially after mitochondrial Ca^{2+} loading. Ruthenium red (RR), a potent inhibitor of the mitochondrial Ca^{2+} uptake, prevented this effect, supporting the importance of Ca^{2+} entrance into the mitochondria in the observed effect. Our results also showed that the decrease of ΔΨ elicited by 3HTA after Ca^{2+} addition was due to a non-selective membrane permeabilization caused by mPTP opening, since ΔΨ decrease was accompanied by mitochondrial swelling and both were inhibited by CsA plus ADP, classical inhibitors of the mPTP by binding to cyclophilin D and to ANT, respectively.

The mPTP formation permits the release of small mitochondrial components to the cytosol. Thus, the reduction of NAD(P)H matrix content may result from its release from the mitochondria through the mPTP formation or from increased NAD(P)H oxidation leading to impairment of mitochondrial redox homeostasis (Kehrer and Lund, 1994; Kowaltowski et al., 2001b; Le-Quoc and Le-Quoc, 1989). We also found that 3 HTA reduced the mitochondrial Ca^{2+} retention capacity, probably through mPTP opening once Ca^{2+} release was prevented by CsA plus ADP.

Regarding to the mechanisms involved in mPTP induction, it has been shown that fatty acids provoke oxidative stress (Leipnitz et al., 2010; Schuck et al., 2009a; Tonin et al., 2010b) and uncoupling of oxidative phosphorylation (Samartsev et al., 2011; Schuck et al., 2009b; Tonin et al., 2013; Tonin et al., 2010a), which have been pointed as important mechanisms for mPTP opening (Kowaltowski et al., 2001a; Zago et al., 2000; Zoratti and Szabo, 1995). In our study, we found an induction of H_2O_2 production caused by 3 HTA only after Ca^{2+} addition showing that, in this case, ROS production was triggered by mPTP opening. Increase H_2O_2 production was prevented by RR and by CsA plus ADP confirming the influence of mPTP formation leading to H_2O_2 production, as

previously found in other pathological situations (Penna et al., 2013; Zorov et al., 2006). We cannot rule out that 3 HTA-elicited H_2O_2 augment could be due to a reduction of glutathione peroxidase activity caused by the decrease of NAD(P)H, which is a substrate of this potent antioxidant enzyme.

It was also observed that 3 HTA reduced mitochondrial cytochrome *c* immunocontent in Ca^{2+} loaded mitochondria most likely as a consequence of extensive swelling and rupture of outer mitochondrial membrane. The prevention of this effect caused by RR or CsA plus ADP support this hypothesis. In addition, 3 HTA and 3 HPA provoked a reduction in ATP levels possibly highlighting the uncoupling effect induced by these fatty acids impairing the maintenance of $\Delta\Psi$ necessary to ATP biosynthesis (Brand and Nicholls, 2011). However, other mechanisms caused by these fatty acids may underlie the reduction of ATP levels. When Ca^{2+} was added to the media, a further decrease in ATP content was observed probably caused by the collapse of $\Delta\Psi$m and/or loss of adenine nucleotides to the external media through mPTP formation (Bernardi and Rasola, 2007; Rasola and Bernardi, 2011). Considering that the loss of cytochrome *c* to the extra mitochondrial space activates the caspase cascade, ou r results may indicate that the accumulating compounds probably cause apoptotic cell death, although the low availability of ATP may lead to cell necrosis (Eguchi et al., 1997).

As previously found in the heart, the effects of the LCHFA were dependent on the carbon-chain length so that the longer the carbon chain the stronger were these effects, as verified for mitochondrial swelling and the reduction of ATP content.

At present, it is difficult to determine pathophysiological relevance of our present data since the concentrations of the LCHFA in the heart and brain of patients with MTP and LCHAD deficiencies are still unknown. However, it should be stressed that the significant alterations of the biochemical parameters verified in our present study were achieved with low micromolar concentrations (10 μM and higher) of these compounds, similar to those found in plasma of the affected patients (Costa et al.,

1998). Furthermore, it is also feasible that the concentrations of the accumulating LCHFA may dramatically increase in these patients during metabolic crises in which fatty acids are released from the adipose tissue and cannot be metabolized due to the blockage of the enzymatic steps catalyzed by MTP or LCHAD (Costa et al., 1998; Halldin et al., 2007; Scriver, 2001). The present data therefore reinforce the hypothesis that accumulation of fatty acids and their derivatives may be involved in the cardiomyopathy and neurological dysfunction found in these diseases (Ventura et al., 2007).

Taken together, we verified that the accumulation of LCHFA might have deleterious consequences for mitochondrial energy homeostasis in heart and brain. In case our present *in vitro* findings are confirmed *in vivo* in animal experiments and also in tissues from patients affected by these fatty acid oxidation defects, it is proposed that mitochondrial dysfunction may contribute to the cardiac and neurological alterations presented by MTP- and LCHAD- deficient patients. We therefore suggest that mPTP inhibitors may be potentially interesting as therapeutic candidates for the prevention of mitochondrial alterations in these diseases.

In the last chapter, we evaluated the effects of LCHFA on oxidative stress parameters on rat heart. We observed that the LCHFA 3 HDA, 3 HTA and 3 HPA did not alter TBA-RS, a lipid peroxidation marker, as well as the protein oxidative damage indicators sulfhydryl and carbonyl content. Furthermore, the levels of reduced glutathione were not altered in the presence of these fatty acids. Differently from what happened in rat brain (Tonin et al., 2010b), LCHFA were unable to induce oxidative stress in rat heart under our experimental conditions. This was also consistent with our findings showed in chapter III in which these fatty acids decreased hydrogen peroxide production in heart mitochondrial preparations. The difference observed between tissues could be related to cell

antioxidant properties and biochemical composition that makes the brain more susceptible to oxidative damage, as previously mentioned.

Taken together, we showed in the present investigation that the major fatty acids and derivatives accumulating in MCAD, LCHAD and MTP deficiencies disrupt energy metabolism and redox homeostasis. We also demonstrated that fibroblasts from MCADD and MTPD patients are under chronic oxidative stress when cultured under stress conditions (media with Gal or Gal plus Palm) and that these cells from MTP patients are more prone to death. It is therefore presumed that these pathomechanisms may underlie the toxicity of these compounds towards the heart and brain and contribute at least partially to the cardiac and brain dysfunction observed in patients affected by these disorders. So, we presume that the deleterious effects of these fatty acids associated to hypoglycemia, especially during episodes of metabolic decompensation, may act synergistically to cause some of the clinical manifestations characteristic of these disorders.

4. Conclusions

4.1 Medium-chain acylcarnitines (MCAC) accumulated in MCADD disrupt redox homeostasis in rat cerebral cortex

- Hexanoylcarnitine (HC), octanoylcarnitine (OC) and decanoylcarnitine (DC) induced lipid oxidative damage (increased thiobarbituric reactive substances - TBA-RS- levels) and decreased the antioxidant defenses (reduction of reduced glutathione- GSH- levels) in cerebral cortex from 30-day old rats.

- DC also induced protein oxidative damage (increase of carbonyl formation and decrease of sulfhydryl content) in cerebral cortex of young rats.
- The increase of TBA-RS levels and the decrease of GSH levels induced by DC were prevented by trolox (TRO) and melatonin (MEL) indicating the involvement of the reactive species peroxyl and hydroxyl that are mainly scavenged by these free radical scavengers.
- A strong inverse correlation between TBA-RS and GSH values caused by DC indicates that the decrease of the brain antioxidant defenses and the induction of lipid peroxidation are dependent effects probably secondary to DC-induced free radical generation.
- Hexanoic acid (HA), L-carnitine and hexanoylglycine did not change TBA-RS levels, carbonyl and sulfhydryl content as well as GSH concentrations, differently for what was observed for HC, implying that HC is more toxic than HA and the other derivative compounds.
- In summary, the MCAC accumulating in MCADD induce oxidative stress *in vitro*.

4.2 Superoxide generation and cell death induction in cultured fibroblasts from patients affected by MCADD and MTPD under different cultured conditions

- Fibroblasts from MTPD patients presented higher levels of superoxide and cell death under standard growing conditions as compared to controls, suggesting a chronic exposure to oxidative stress in these patients.
- Under metabolic stress conditions, superoxide levels were similarly increased in fibroblasts from both groups of patients (MCADD and MTPD) compared to controls.
- Cells from MTPD patients submitted to metabolic stress had a similar rate of cell death compared to basal conditions.

- Cells from MCADD patients submitted to different cultured conditions (standard and metabolic stress) did not present significantly higher cell death.
- No correlation was observed between distinct mutations presented by patients' fibroblasts, implying a lack of correlation between genotype and biochemical phenotype.
- Significant correlation was found between superoxide production and cell death in fibroblasts from MTPD patients.
- In summary, fibroblasts from MTPD and MCADD patients present higher superoxide production, whereas cell death only occurred in fibroblasts from MTPD patients.

4.3 Long-chain 3-hydroxyfatty acids (LCHFA) accumulating in LCHAD and MTP deficiencies uncouple oxidative phosphorylation in mitochondria of rat heart

- LCHFA, 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids increased state 4 of mitochondrial respiration and decreased the respiratory control ration (RCR) in rat heart
- 3-Hydroxydodecanoic acid (3HDDA), 3HTA and 3 HPA dissipated mitochondrial membrane potential ($\Delta\Psi$) and decreased the matrix pool of NAD(P)H and the H_2O_2 production.
- These findings indicate that these fatty acids act as uncouplers of the oxidative phosphorylation.
- The dissipation of the $\Delta\Psi$ and mitochondrial swelling induced by 3 HTA in the presence of Ca^{2+} was prevented by cyclosporin A (CsA) suggesting the involvement of the mitochondrial permeability transition pore (mPTP) in such effect.
- 3 HTA decreased ADP/O ratio, indicating an ineffective oxidative phosphorylation.
- In summary, LCHFA compromise oxidative phosphorylation in rat heart *in vitro.*

4.4 LCHFA induce mitochondrial permeability transition pore opening in cerebral cortex from rats

- 3 HTA dissipated $\Delta\Psi$ and reduced matrix NAD(P)H pool and increased H_2O_2 levels in rat cerebral cortex.
- 3HDA, 3HTA and 3HPA induced mitochondrial swelling in Ca^{2+} loaded mitochondria.
- 3 HTA released cytochrome *c* from mitochondrial matrix, indicating apoptosis induction.
- 3 HTA and 3 HPA reduced ATP content.
- These effects were prevented by ruthenium red (RR) and by CsA plus ADP, indicating mitochondrial permeability transition pore (mPTP) involvement.
- In summary, LCHFA, particularly 3HTA, induce mPTP in rat cerebral cortex *in vitro*.

5. Perspectives

- Investigate the effect of medium-chain acylcarnitines on parameters of redox homeostasis in, liver and muscle of young rats.
- Investigate markers of oxidative stress and cellular antioxidant response as well as the mechanisms of cell death in fibroblasts from medium-chain acyl-CoA dehydrogenase (MCADD) and mitochondrial trifunctional protein (MTPD) deficient patients under standard and metabolic stress conditions.
- Investigate markers of oxidative stress and cellular antioxidant response in blood from MCADD and MTPD patients.
- Evaluate the i*n vitro* effects of long-chain 3-hydroxy fatty acids (LCHFA) on energy and redox homeostasis in liver and muscle from young rats.
- Evaluate the mechanisms involved in mitochondrial permeability transition (mPTP) opening induced by LCHFA in mitochondrial preparations of heart and brain from young rats.

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